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Original Paper

Changes of cadmium concentration and expressions of candidate genes for the cadmium transporter in intestine, after short term low dose exposure.

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Cadmium (Cd) is a highly toxic metal and food is the major source of Cd exposure for most people. Divalent metal transporter 1 (DMT1) is thought the main Cd transporter *in vivo*. However, in our previous study we found an unknown Cd pathway other than DMT1. Thus, additional *in vivo* study are needed to explore the Cd pathway.

Seven-week-old ICR mice were given 1 ppm $CdCl_2$ aq in drinking water for zero to five days ad libitum. The concentrations of Cd in liver, kidney, and intestinal epithelium were measured. Gene expression changes in intestinal epithelium were analyzed by microarray and real time PCR analyses. Intestinal epithelial Cd concentrations were increased up to day2, but from day3 they decreased irrespective of an increase in total Cd intake. The microarray and real-time PCR analyses could not demonstrate Cd concentration-specific alterations in intestinal epithelium after this short-term and low-dose Cd exposure. However some gene expression changes associated with unknown Cd uptake pathways through the intestinal brush border may have played a role in the observed specific Cd concentration changes. It may be possible that metallothionein plays a role in the Cd uptake pathway.

Keywords: Cadmium uptake; intestine; Gene expression; DMT1; Zip; Metallothionein

INTRODUCTION

Cadmium (Cd) is a highly toxic metal that may cause nephropathy and osteomalacia, and it has been recognized as an environmental contaminant that may enter the food chain. Food is the major source of Cd exposure for people who are not smokers. Thus, the mechanisms of intestinal Cd absorption need to be elucidated to gain an understanding of Cd toxicity to the human body. High blood Cd levels have been observed in pregnant women [1] and in iron (Fe) deficiency [2]. It is also known that intestinal uptake of Cd is inhibited by Fe [3]. Thus, the processes of Cd and Fe uptake are thought to be overlapping. Needless to say Fe is an essential metal and it is needed for almost all cellular functions, such as oxygen transport, energy production, and cell proliferation. Fe is absorbed in the intestine, especially in the duodenum.

Divalent metal transporter 1 (DMT1) was reported to be located at the brush border of human [4] and rat [5] duodenum by immunohistochemistry. It is thought to be the main transporter for absorption of Fe through the brush-border membranes of the mammalian intestine [6]. In a study of *Xenopus laevis* oocytes, it was shown that DMT1 transports both Fe and Cd [6,7]. DMT1 expression is up-regulated in subjects with Fe-deficiency [8,9] and this coincides

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with increased Cd absorption in humans with Fe-deficiency [2]. Therefore, DMT1 is a candidate for transporting Cd and Fe at the brush border membrane of the duodenum. Recent studies have shown that DMT1 transports Fe and Cd *in vitro* [7] and a few *in vivo* studies have been reported, however the latter studies were not able to differentiate transport by DMT1 from that of other transporters [10,11,12].

The MK/Rej-$^{mk}/_{mk}$ mouse (*mk/mk*) is an animal model of hereditary microcytic anemia[13]. A mutation (G185R) in the TM domain 4 of DMT1 in *mk/mk* mice causes the severe microcytic anemia, associated with impairment of Fe absorption in the intestine [14,15,16]. In *mk/mk* mice, a non-functional DMT1 protein is over-expressed and is not properly targeted to the brush border of the intestinal epithelium [17].

In our previous study we compared Cd absorption in *mk/mk* mice and Fe-deficient wild type (WT) mice to clarify the role of DMT1 in intestinal Cd absorption *in vivo*. In *mk/mk* mice the mutated G185R DMT1 had lost its function, but Cd accumulation was unchanged. It is therefore possible that an unknown Cd pathway plays a role in Cd intestinal absorption in Fe deficiency *in vivo*. Therefore, more *in vivo* studies are needed for the understanding of the intestinal Cd accumulation, particularly that due to an unknown Cd pathway.

It is known that intestinal Cd uptakes increase in Zink (Zn) depleted rats [18], so the *in vivo* uptake pathways for Zn and Cd may be related, or even identical.

The aim of this study is to explore the unknown Cd pathway by measuring changes in organ Cd concentration and mRNA expression of Cd pathway candidates during low-dose, short-term Cd exposure.

MATERIALS AND METHODS

Animals and diets

Female ICR mice, aged 7weeks, were purchased from Sankyo Labo Service Co, (Tokyo, Japan). The mice received a normal diet of CE-2 (CLEA Japan Inc, Tokyo, Japan) and tap water ad libitum before experiments were started, and they were housed at 25°C with 50% humidity and a 12h light/dark cycle (0800-2000 light and 2000-0800 dark). All animal care use and procedures in this study were based on guidelines of the National Institutes of Health (NIH), and the principles and guidelines of the Kyoritsu University of Pharmacy.

Experimental design

Seven-week-old ICR mice were used. From the 7th week, all mice were given 1 ppm $CdCl_2$ aq in drinking water for zero to five days ad libitum (day0; n=3, days 1, 2 and 3; n=6, days 4 and 5; n=3). After Cd administration, mice were anesthetized with sevoflurane. Their abdomens were surgically opened, and the liver, kidney and intestinal epithelium were extirpated.

Cadmium analyses of liver, kidney and small intestine

Cd status in the body was evaluated by determining the concentrations of Cd in liver, kidney, and intestinal epithelium. These tissues were subjected to wet digestion by nitric acid and perchloric acid. Digestion of 0.1 – 0.2 g of tissue was carried out with a mixture of 2.0 mL of 60% HNO_3 and 0.1 mL of $HClO_4$ in a Pyrex tube at 90 °C for 1h, 130 °C for 2h and 200 °C for 1.5 h. The digested samples were diluted with ultra pure water and used for measuring Cd and Fe concentrations. All glassware was thoroughly washed and then soaked overnight in 30% nitric acid, and rinsed with ultra pure water before use. Tissue Cd concentrations were measured using a flameless-type atomic absorption spectrophotometer (Shimadzu AA-660G, Shimadzu corp., Kyoto, Japan) at 1100°C for 3s at 228.8 nm. Cd standard solution for atomic absorption analysis (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used for determining a standard curve and for quality control.

DNA microarray analyses of transporters in small intestine

Gene expression changes in intestinal epithelium of day0 and day3 mice were analyzed by using the AceGene® Mouse Oligo Chip (Hitachi Software Engineering Co.,Ltd., Kanagawa, Japan). The microarray analysis was performed by DNA Chip Research Inc (Kanagawa, Japan).

Real time PCR analyses of transporters in small intestine

The mRNA levels of Zip1, Zip2, Zip4, DMT1 and metallothionein-2 (MT-2) in intestinal epithelium of

the mice were analyzed by real time PCR analyses. Mice were treated with drinking water that included Cd as described above (day0, 1, 2 and 3; n=6). Mice were sacrificed as described above and epithelia from the small intestine were isolated as described previously. Total RNA was extracted from scraped small intestinal epithelium by using ISOGEN (NipponGene, Tokyo, Japan), according to the manufacturer's instructions and stored at –80 °C until required. Total RNA was purified using RNeasy® Plus Mini Kit (QIAGEN K.K., Tokyo, Japan) and used for real time reverse transcription-polymerase chain reaction (real time RT-PCR). Reverse transcription was performed with 2 μg of purified RNA using a TaqMan® Reverse Transcription Reagents (Applied Biosystems Japan, Tokyo, Japan), according to the manufacturer's instructions. The cDNA was amplified in TaqMan® Universal PCR Master Mix (Applied Biosystems Japan, Tokyo, Japan) from the RT products with TaqMan Gene Expression Assays (Applied Biosystems Japan, Tokyo, Japan). The amplified PCR products were normalized by the GAPDH levels.

Statistical analyses

All values are expressed as means ± SE. Statistical evaluation of the data were at first examined for normal distributions by the Kolmogorov-Smirnov test. Differences between groups were analyzed statistically using one-way analysis of variance (ANOVA). When differences were detected, means were tested with the LSD test for differences between groups. Statistical significance was set at $P<0.01$ and $P<0.05$.

RESULTS

The body weights of the mice did not change dramatically, although the body weights of day2 mice were significantly lower than those of day3 mice. No significant changes were observed in liver, kidney or intestinal epithelium weight per body weight after Cd administration. The total Cd intakes were increased over time (days), but there was no significant difference between daily Cd intakes.

Hepatic and renal Cd concentrations were increased as the total Cd intakes increased over time (Table1). Hepatic Cd concentrations of day1, 4 and 5 mice were significantly higher than those of day0 mice. Renal Cd concentrations of day3, 4 and 5 mice were significantly higher than those of day0 mice (Fig.1 A).

Intestinal epithelial Cd concentrations were increased up to day2. After day3, Cd concentrations in intestinal epithelium were decreased irrespective of an increase in total Cd intake. The Cd concentration in intestinal epithelium on day2 was significantly higher than on days 0, 1, 3, 4 and 5 (Fig.1 B).

Table 1 Body, organ weight and Cd intake.

	Day 0 (n=3)	Day 1 (n=6)	Day 2 (n=6)	Day3 (n=6)	Day 4 (n=3)	Day 5 (n=3)
Body weight (g)	28.33 ± 0.32	27.55 ± 0.47	26.92 ± 0.74[d]	28.85 ± 0.42	27.83 ± 0.32	28.13 ± 0.50
Liver wet weight/ body weight (mg / g)	47.28 ± 3.73	54.83 ± 3.09	55.86 ± 2.83	51.77 ± 0.32	56.39 ± 5.83	55.03 ± 2.58
Kidney wet weight / body weight (mg / g)	6.89 ± 0.33	7.05 ± 0.20	7.45 ± 0.48	6.98 ± 0.09	7.26 ± 0.36	7.36 ± 0.18
Intestine wet weight / body weight (mg / g)	33.58 ± 2.33	35.49 ± 2.80	34.79 ± 4.46	36.71 ± 1.26	42.48 ± 5.40	38.95 ± 4.44
$CdCl_2$ aq intake (mL/day)	0	5.92 ± 0.62	7.23 ± 1.38	6.99 ± 0.69	8.83 ± 1.41	8.75 ± 0.96

Data are mean ± se.
[d] : $p<0.05$ compared with day [3]

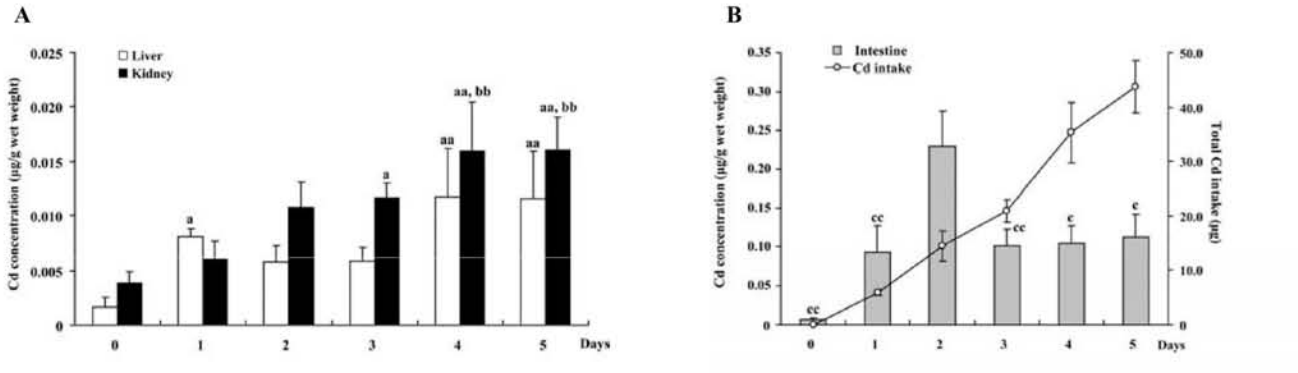

Fig.1 Tissue Cadmium concentrations in liver, kidney (A) and intestine (B) and total Cadmium intakes (B).

A. Cd concentrations in mouse liver (white bar) and kidney (black bar). Hepatic Cd concentrations of day1 mice (p<0.05) and days 4 and 5 mice (p<0.01) were significantly higher than those in day0 mice. Renal Cd concentrations of day4 and day5 mice were significantly higher than those of day0 and day1 mice (p<0.01). Points shown are means ± SE. (days 1, 2 and 3: n =6; days 0, 4 and 5: n=3) ^{aa} : p<0.01 vs. day0, ^{a} : p<0.05 vs. day0, ^{bb} : p<0.01 vs. day1, ^{b} : p<0.05 vs. day1

B. Cd concentration in mouse intestinal epithelium (gray bar) and total Cd intake (line). Cd concentration in intestinal epithelium of day2 mice was significantly higher than that of days 0, 1 and 3 mice (p<0.01) and days 4 and 5 mice (p<0.05). Total Cd intake was increased over time (days). Points shown are means ± SE. (days 1, 2 and 3: n =6; days 0, 4 and5: n=3) ^{cc} : p<0.01 vs. day2, ^{c} : p<0.05 vs. day2

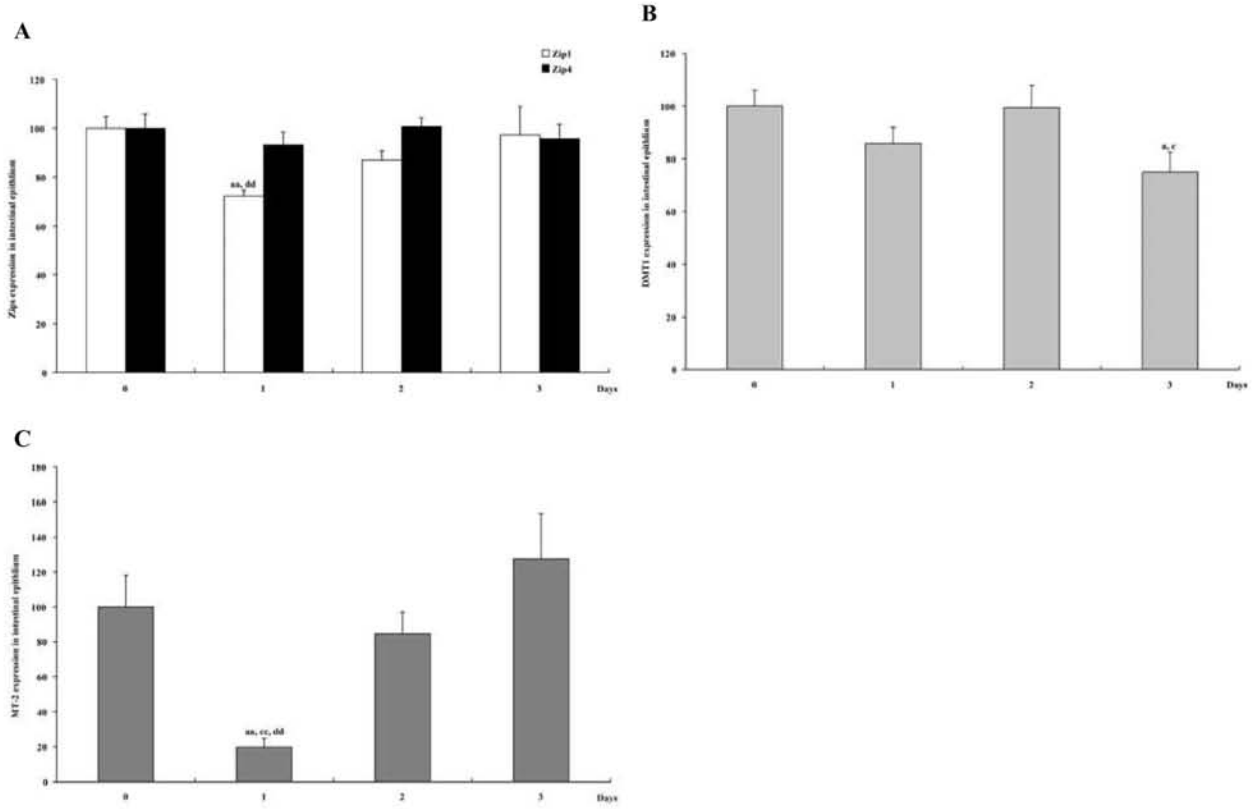

Fig.2 Gene expressions of ZIP1, Zip4 (A), DMT1 (B) and MT-2 (C) in mouse intestinal epithelium.

A. Expression of Zip1 (white bar) and Zip4 (black bar) mRNA in mouse intestinal epithelium. Expression of Zip1 mRNA in intestinal epithelium of day1 mice was significantly lower than that in day0 and day3 mice (p<0.01). Points shown are means ± SE. (n =6) ^{aa} : p<0.01 vs. day0, ^{dd} : p<0.01 vs. day3

B. Expression of DMT1 mRNA in intestinal epithelium of day3 mice was significantly lower than that in day0 and day 2 mice (p<0.05). Points shown are means ± SE. (n =6) ^{a} : p<0.05 vs. day0, ^{c} : p<0.05 vs. day2

C. Expression of MT-2 mRNA in intestinal epithelium of day1 mice was significantly lower than that in days 0, 2 and 3 mice (p<0.01). Points shown are means ± SE. (n =6) ^{aa} : p<0.01 vs. day0, ^{cc} : p<0.01 vs. day2, ^{dd} : p<0.01 vs. day3

Table 2 Summary of microarray data. Altered gene expressions and changes of Cd pathway candidate genes in intestinal epithelium of day0 and day3 mice.

Accession Number	Gene Symbol	Day0 Intensity	Ratio (Ch1/Ch2)	Description
up-regulated gene				
U28244	Pla2g2a	55	16.69	type ii phospholipase a2
NM 026097	Rffl	5	9.13	metal ion binding
AF361350	Cacng8	14	8.08	voltage-dependent calcium channel cacng8
ENSMUST 00000041928	Rnf20	7	7.84	metal ion binding
AK002567	Mt2	102	7.36	metallothionein 2, metal ion binding
down-regulated gene				
NM 011752	Zfp259	135	0.17	zinc finger protein 259
BC023060	Efemp1	30	0.22	protein binding,calcium ion binding
NM 007673	Cdx2	143	0.23	transcription factor activity
NM 009788	Calb1	161	0.24	vitamin D, calcium ion binding calbindin-28k, calb1
NM 023137	Ubd	863	0.26	ubiquitin d
solute carrier family 11				
NM 013612	Slc11a1	1576	0.85	membrane iron ion transporter
NM 008732	Slc11a2	279	1.48	membrane iron ion transporter, DMT1
BC019137	Slc11a2	2055	0.89	membrane iron ion transporter, DMT1
solute carrier family 39				
ENSMUST 00000047726	Slc39a2	237	0.83	zinc ion transporter activity
ENSMUST 00000004676	Slc39a2	29	2.89	zinc ion transporter activity
NM 134135	Slc39a3	136	0.93	metal, zinc ion transporter activity
AK005535	Slc39a4	3041	0.86	zinc ion transporter activity
AK007473	Slc39a5	856	1.25	zinc ion transporter activity
AB071697	Slc39a6	195	1.11	metal ion transporter activity
NM 008202	Slc39a7	496	1.04	metal ion transporter activity
NM 026228	Slc39a8	105	1.08	metal ion transporter activity
ENSMUST 00000037267	Slc39a10	379	0.72	metal ion transporter activity
ENSMUST 00000027131	Slc39a10	37	2.66	metal ion transporter activity
BC019647	Slc39a11	129	0.73	metal ion transporter activity
AK007986	Slc39a11	95	1.22	metal ion transporter activity
ENSMUST 00000036169	Slc39a12	879	0.90	unknown
AK003191	Slc39a13	1779	0.90	metal ion transporter activity
BC021530	Slc39a14	341	1.11	unknown
metallothioneins				
NM 013602	Mt1	631	0.88	metallothionein 1, metal ion binding
AK002567	Mt2	102	7.36	metallothionein 2, metal ion binding
NM 013603	Mt3	17	2.04	metallothionein 3, metal ion binding
NM 008631	Mt4	155	0.90	metallothionein 4, metal ion binding

The results of the microarray analysis of altere expressions and changes in Cd pathway candidate genes in intestinal epithelium in day0 and day3 mice are shown in Table2.

The mRNA expression of Zip1 on day 1 was significantly lower than on day0 and day3. No significant changes were observed in mRNA expression of Zip4 (Fig. 2 A). No mRNA expression of Zip2 was detected by real-time PCR analysis. The mRNA expression of DMT1 in day3 mice was significantly lower than on day0 and 2 (Fig.2 B). The mRNA expression of MT-2 on day1 was significantly lower than on day0, 2 and 3 (Fig.2 C).

DISCUSSION

In this study we investigated the Cd concentration and gene expression of Cd transporters in mouse intestine after short-term, low-dose exposure.

The body weights of day2 mice were significantly lower than those of day3 mice. However, no significant changes were observed in liver, kidney or intestinal epithelium weights per body weight after Cd administration during the experimental days. The intake of $CdCl_2$ aq was the same for all groups throughout the experimental days. Inhibition of body weight gain or organ weight loss were not observed, and daily Cd intake was not changed. Thus administration of 1 ppm $CdCl_2$ aq was nontoxic to mice in this experiment.

The hepatic and renal Cd concentrations were slowly increased as total Cd intake was increased. The Cd concentrations in the intestinal epithelium were increased from day0 to day2. However on day3, the Cd concentration was decreased to the same level as on day1 and stayed at the lower level. Nevertheless, the daily Cd intake was unchanged, and the total Cd intake continued to increase by time. From these data two conceivable hypotheses can be proposed: according to the first, there was an increased Cd transport from the intestinal epithelium through the basolateral membrane to blood circulation; according to the second, there was a decreased Cd transport from the intestinal brush border to the intestinal epithelium through the brush border membrane. If the first hypothesis were correct, large amounts of Cd (about half of the amounts present inside the intestinal epithelium) would be transported into the bloodstream between day2 and day3. Considering that the Cd concentration in the intestinal epithelium was ten times higher than those of other organs, the Cd concentrations in the other organs, particularly that of the liver, should be increased on day3; however we found that the hepatic Cd concentration was not increased on day3. Therefore, the former hypothesis was rejected. Thus these results suggested that the expression of another Cd pathway, uptake from the intestinal brush border to the intestinal epithelium was changed.

According to the microarray analysis, there were no changes in expression of candidates for the Cd uptake pathway except Zip2. Although the gene expression changes of Zip2 were detected by microarray analysis, they could not be detected by real-time PCR analysis. The Zip2 mRNA expression was lower than the limit of detection by real-time PCR analysis. Thus Zip2 expression is very limited and its contribution to Cd uptake must be insignificant, therefore limitations of Cd transport to intestinal epithelium were not mediated by Zip2. Although some Zip1 gene expression changes were observed on day1, they were not large, and the expression had recovered on day2, thus Zip1 cannot be responsible for limitations of Cd transport to the intestinal epithelium. No Zip4 gene expression changes were observed, so limitations of Cd transport to the intestinal epithelium were not mediated by Zip1 nor Zip4. DMT1 gene expression changes were observed on day3, but the level was almost the same as on day1. Considering that the Cd concentrations were increasing from day0 to day2, the DMT1 expression changes cannot explain the limitations of Cd transport to the intestinal epithelium.

In this study we compared gene expressions in intestinal epithelium of day0 and day3 mice by microarray analysis. The intestinal epithelium Cd concentration was changed from day2 to day3, thus, it is possible that gene expression changes happened before day3. Therefore, we compared gene expressions in intestinal epithelium of day0 and day1 or day2 mice.

The large MT-2 gene expression was decreased on day1, day2 and day3. MT-2 expression then increased and recovered to the same expression level as that of the control. Although the MT-2 expression changes were quite large, they could not explain the

limitations of Cd transport to the intestinal epithelium. However, because MT-2 expression changes were observed, it may be possible that metallothionein plays a role in the Cd uptake pathway. Therefore more *in vivo* studies are needed to clarify the role of metallothionein in Cd uptake pathways.

In conclusion, intestinal epithelium Cd concentration showed specific alterations during short-term, low-dose Cd exposure. Expression changes of an unknown Cd uptake pathway through the intestinal brush border may have played a role in these specific alterations. It may be possible that metallothionein plays a role in the Cd uptake pathway.

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