INTRODUCTION
Cadmium (Cd) is one of the most toxic of the heavy metals, and as an environmental pollutant it poses a threat to animal and human health (Jarup, 2002). Cd is present in food or water, and accumulates in human or animal tissues especially the liver and kidney. It has been reported that exposure to low levels of Cd may result in renal damage and osteoporosis, which are considered to be the major adverse health effects of Cd exposure in laboratory animals (Brzoska and Moniuszko-Jakoniuk, 2004; Honda et al., 2003; Ohta et al., 2000).

Metallothionein (MT) plays an important role in the detoxification of heavy metals such as Cd (Bremner, 1987). The MT was first isolated in 1957 from the cortex of horse kidney as a Cd-binding protein (Margoshes and Vallee, 1957), and was first identified in 1960 (Kagi and Vallee, 1960). In cases of oral Cd administration, Cd bound by MT (MT-Cd) is taken up in an intact form by intestinal cells and transported selectively to the kidneys (Ohta and Cherian, 1991). Chan and Cherian (1993) reported that in Cd pre-treated rats, pregnancy can result in mobilization of MT-Cd in the liver, which is then transferred to the kidney and placenta through the blood. Several researchers have postulated that transport of Cd to the fetus is restrained by MT in the placenta (Goyer, 1991; Goyer et al., 1992). Some studies were reported that the MT in rat has the effect of Cd toxicity on renal function, that chemical forms of Cd was important for Cd toxicity in placenta (Arizono et al., 1981; Hazlehoff Roelfzema et al., 1989; Tekin, 2011). However, the precise role of MT in the mechanism of Cd transport from mother to fetus is yet to be fully elucidated.

Health effects of low-level cadmium intake and the role of metallothionein on cadmium transport from mother rats to fetus
Yasuhiro Nakamura1, Ken-ichi Ohba1, Keiji Suzuki2 and Hisayoshi Ohta1

1Department of Environmental, Occupational Health and Toxicology, School of Allied Health Sciences, Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
2School of Health Sciences, Faculty of Medicine, Gunma University, 3-39-15 Shouwa-machi, Maebashi, Gunma 371-8511, Japan

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ABSTRACT — Female Wistar rats were given Cd (as CdCl2) at a dose of 0, 1, 2, and 5 mgCd/kg/day by gastric tube daily for 6 consecutive days each week for 10 weeks. After the birth, newborn rats were sacrificed on day 1 and at 4 weeks. Mother rats were sacrificed after 4 weeks of lactation. The concentrations of Cd in uterus and placenta, and metallothionein (MT) in the uterus of mother rats were determined. The concentrations of Cd in kidney and liver of newborn rats were also determined. Expression of iso-MT genes (I, II, and III) in the uterus of mother rats was measured using RT-PCR. The Cd concentration in the liver of newborn rats at the first day after birth was higher than in the kidney, while the concentration in the kidney of newborn rats at the fourth week after the birth was significantly higher than in the liver. The uterine MT concentration increased with accumulation of Cd; however, the MT concentration did not increase enough to prevent Cd transport to the fetus. On the other hand, it was considered that more Cd was transported as the chemical form of non-MT-Cd from mother rat, and accumulated in the liver rather than kidney of the fetus. Based on analyses of the Cd distribution in the liver and kidney of newborn rats, we speculate that MT in the uterus and placenta does not play a significant role in preventing Cd transport through the placenta from the uterus to the fetus.

Key words: Metallothionein, Cadmium, Placenta, Uterus

Correspondence: Hisayoshi Ohta (E-mail: hohta@kitasato-u.ac.jp)
not clear. We therefore examined the role of MT on the transport of Cd between mother and fetus in rats.

**MATERIALS AND METHODS**

**Experimental animals**

The present experimental study was performed by approval of an animal ethical committee of Kitasato university. Female Wistar rats (6 weeks old) were obtained from the Nihon Kurea Co. Female rats were separated into 4 experimental groups containing 5 animals each. Rats were administered Cd (as CdCl₂) at doses of 0, 1, 2, or 5 mg Cd/kg/day. The CdCl₂ was administered by gastric tube daily for 6 consecutive days each week from 3 weeks prior to mating until the fourth week of lactation. Mother rats were sacrificed after 4 weeks of lactation. Four rats of both sexes per litter were selected on the first day after birth from a mother rat in each experimental group. Four of the remaining newborn rats of both sexes were lactated over the following 4 weeks. Blood sample of mother rats was collected via cardiac puncture under Nembutal anesthesia, after which the animals were sacrificed by exsanguination. Blood remaining in the organs was immediately washed out by perfusion using an ice-cold saline solution, after which various organs such as liver, kidneys, and uterus were removed. Newborn rats were sacrificed by heart blood sampling under diethyl ether anesthesia, and the liver and kidneys were removed immediately. An additional group of female rats (n = 5) was prepared and treated in the same manner as the other experimental groups. These animals were sacrificed by heart blood sampling under pentobarbital anesthesia on the 19th day or the 20th day since pregnant. The placenta and fetuses were removed immediately after blood remaining in the organs was washed out by perfusion using ice-cold saline solution.

**Determination of Cd**

The concentration of Cd in the uterus and placenta of mother rats and in the kidney and liver of newborn rats at the first day and at the fourth week after birth was determined using flame or flameless atomic absorption spectrophotometry (Hitachi Zeeman 180) using the wavelengths 228.8 nm for Cd. Additionally, the amount of Cd not bound by MT (nonMT-Cd) was calculated as the difference between the T-Cd and MT-Cd concentrations according to previously reported methods (Nomiyama and Nomiyama, 1982; Onosaka et al., 1978).

**Reverse transcriptional (RT)-PCR**

RNA was isolated using an SV Total RNA Isolation System (Promega, Tokyo, Japan). The RT reaction was performed in a mixture containing 12.5 μl of Access Quick Master Mix (Promega), 0.1 μg of total RNA, 10 pmol of Oligo (dT) primer (TOYOBO, Osaka, Japan), and 1 μl of AMV reverse transcriptase (Promega) in a total volume of 20 μl. PCR amplification involved 30 cycles of denaturation at 94°C for 2 min, annealing at 65°C for 1 min, and extension at 72°C for 30 sec, followed by reverse transcription at 48°C for 45 min. The PCR primers for rat MT-I, MT-II and MT-III were as follows: MT-I (forward primer: 5'-ATG GAC CCC AAC TGC TCC TGC TCC ACC-3’ and reverse primer: 5’-TCA GGC ACA GCA CGT GCA CTT GTC-3’), MT-II (forward primer: 5’-ATG GAC CCC AAC TGC TCC TGT GCC ACA-3’ and reverse primer: 5’-TCA GGC GCA GCA GCT GCA CTT GTC-3’) and MT-III (forward primer: 5’-ATG GAC CCT GAG ACC TGC CCC-3’ and reverse primer: 5’-TCA CTG GCA GCA GCT GCA TTG C-3’). The PCR products were resolved using agarose gel electrophoresis and the intensity of ethidium bromide-stained PCR products was measured using Kodak Digital Science ID Image Analysis Software (Kodak, Tokyo, Japan).

**SDS-PAGE and Immunoblotting**

Proteins isolated from the uterus were subjected to SDS-polyacrylamide gel electrophoresis in an 18% slab-gel. Blotting was carried out by electrophoretic transfer to a PVDF membrane (Amersham Pharmacia Biotech, Tokyo, Japan). The PVDF membrane was incubated with 0.5 % skim milk in TBS (pH 8.0) containing 0.05 % Tween 20 (Tween-TBS) and subsequently incubated overnight with rabbit anti-metal-binding protein primary antibody. The primary antibody used recognizes rat MT, and was the same antibody used in a previously reported study (Ohta et al., 2000). After washing with...
Tween-TBS, the membrane was incubated with an alkaline phosphatase-conjugated secondary antibody for 90 min. Reactive bands were visualized with the chromogenic substrates NBT (nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma Chemical Co., Tokyo, Japan).

**Immunohistochemical studies of MT**

Placental MT protein was stained immunohistochemically as previously described by Nakajima *et al.* (1991). Serial paraffin sections of placenta specimens were incubated in absolute methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activity, and then treated with normal goat serum for 30 min. Sections were then incubated with rabbit anti-MT antibody diluted in phosphate buffered saline, after which the sections were reacted with biotinylated goat anti-rabbit IgG and then processed with a Histofine SAB-PO kit (Nichirei Co., Tokyo, Japan), which detects MT. The MT polyclonal rabbit antibody used in the present study was prepared previously by Nakajima *et al.* (1991), and recognizes both the MT-I and MT-II isoforms of human, rat, or rabbit MT.

**Statistical analysis**

Data were expressed as the mean value with standard deviation. Differences among experimental groups were examined by ANOVA with the PLSD and the Scheffe test at $p < 0.05$.

**RESULTS**

We referred to the results of an epidemiological clinical study of Cd accumulation in the kidney of Japanese who did not show signs of Cd toxicity to estimate the appropriate dose for oral administration in rats in the present study, which corresponded to the daily Cd intake in humans (Yoshida *et al.*, 1998). In addition, we also referred to an experimental study of long-term Cd oral administration to equate rat Cd exposure to human exposure (Ohta *et al.*, 2000). From these studies, we determined that an orally administered dose of 1 mg/kg in this experiment would represent a level similar to the daily Cd intake in humans.

In mother rats exposed to Cd at doses of 1, 2, or 5 mg/kg, the concentration of Cd in the uterus increased significantly in a dose-dependent manner, as shown in Fig. 1A. The concentrations of T-Cd and MT-Cd in the

![Fig. 1. Cadmium concentration in the uterus. Cadmium concentration in the tissue total (A), Cadmium concentration in supernatant fraction (T-Cd) (B), Concentration of cadmium bound by metallothionein in the supernatant fraction (MT-Cd) (C), Concentration of cadmium not bound by metallothionein in the supernatant fraction (nonMT-Cd) (D). * significantly different from control group, $p < 0.05$.](image-url)
uterus were increased significantly at the doses of more than 2 mg/kg (Figs. 1B and 1C). The concentration of nonMT-Cd was calculated from the T-Cd and MT-Cd concentrations as described in the Materials and Methods (Fig. 1D).

A dose-dependent increase in the concentration of Cd was also observed in the placenta of mother rats exposed to Cd at doses of 1, 2, or 5 mg/kg (Fig. 2A). The placental T-Cd concentration also increased significantly in all Cd-exposed groups compared to the control group (Fig. 2B). The concentrations of MT-Cd and nonMT-Cd in the placenta are shown in Figs. 2C and 2D, respectively. The MT-Cd concentration was significantly higher than the control in each Cd-exposed group with the exception of the 1 mg/kg group. Similar results were obtained for the uterus and placenta.

The concentration of Cd in the blood of mother rats was significantly higher in all Cd-exposed groups compared to the control group (Fig. 3). It was thought that the dose-dependent increases in the Cd concentration in the uterus and placenta were due to the dose-dependent increase in Cd in the blood.

It is well-known that MT plays an important role in reducing Cd toxicity. A gradual dose-dependent increase in the expression of iso-MT (I, II, and III) genes was observed in the uterus of Cd-exposed mother rats, and was particularly prominent in the 5 mg/kg group (Fig. 4A). In contrast, the level of MT protein as determined by immunoblotting was lower in the 5 mg/kg group compared to the other Cd-exposed groups, even though the level of uterine MT protein in the 1 and 2 mg/kg groups increased as Cd accumulated in the tissue.
Role of metallothionein in cadmium transport

Fig. 4. Expression of metallothionein genes in the uterus of mother rats. Shows the results of calculation of the density ratio of Total-RNA and RT-PCR product for iso-MTs (I, II, and III) (A). Result of immunoblotting using anti-metal-binding protein (metallothionein) antibodies (B). * significantly different from control group, $p < 0.05$.

Fig. 5. Metallothionein localization in placental tissue after oral cadmium administration (immunohistochemical stain, original magnification $\times 200$). Arrows indicate positive metallothionein staining in placental syncytiotrophoblast cells. Experimental groups; A = Control. B = 1 mgCd/kg. C = 2 mgCd/kg. D = 5 mgCd/kg.
Positive immunohistochemical staining of MT was observed in the syncytiotrophoblast cells of placental tissue in all experimental groups (Figs. 5A, 5B, 5C and 5D). Compared with the control group, reduction of MT staining was found in the 5 mg/kg group (Fig. 5D). Importantly, the morphological change was observed in the 2 and 5 mg/kg groups, and atrophic damage to placental tissue was marked in the 5 mg/kg group (Figs. 5C and 5D). Although the body weight gain in mother rats in the 5 mg/kg group was lower than that of the control group during the gestation period, no significant clinical signs were observed during the treatment period (Data not shown).

Based upon determinations of the Cd concentration in tissues of newborn rats, it appears that Cd is transported from the mother to the fetus in a dose-dependent manner. The Cd concentration in the liver of newborn rats at the first day after birth was higher than in the kidney (Fig. 6); however, the concentration of Cd in the kidney of newborn rats at the fourth week after birth was significantly higher than the concentration in the liver (Fig. 7).

**DISCUSSION**

Cd intake of mother rats caused the dose-dependent increased Cd concentration in uterus and placenta. MT-Cd and nonMT-Cd were also increased in correspondence with the increase of Cd administration dose (Figs. 1 and 2). A statistically significant dose-dependent increase in the Cd concentration in the uterus was observed in all 3 Cd-exposed groups (Fig. 1A). About 50% of Cd was present in the supernatant fraction in the uterus, and almost of Cd amount was present in the supernatant fraction in the placenta in contrast (Figs. 1A, 1B, 2A and 2B). It was considered that Cd had accumulated in placenta was result of increase of Cd concentration in blood of mother rats (Fig. 3).

About 10% of T-Cd concentration in the supernatant fraction was MT-Cd, and the remaining 90% was nonMT-Cd.
Cd in the groups of 2 and 5 mg/kg (Figs. 1B, 1C and 1D). In the placenta, statistically significant increases in the Cd, T-Cd, and nonMT-Cd concentrations were observed for each Cd-exposed group (Figs. 2A, 2B and 2D). However, while a significant increase in the MT-Cd concentration was observed in the 2 and 5 mg/kg groups, no increase was observed in the 1 mg/kg group (Fig. 2C). In addition, similar to the uterus, the nonMT-Cd concentration in the placenta was higher than the MT-Cd concentration. Itoh et al. (1996) reported that placenta received much more Cd accumulation by CdCl\(_2\) injection than that of MT-Cd injection using pregnant mice treated single dose. Also, Chan et al. (1993) reported that an increase in plasma MT during pregnancy aids in the transfer of Cd to the kidney, and that MT-Cd then accumulates selectively in the kidney. Therefore, it was thought that the increase of MT in the placenta resulted after long term Cd exposure. Expression of MT gene in the placenta was probably induced by Cd accumulation. Although MT may participate in trapping Cd in the placenta, considering that the ratio of MT-Cd to T-Cd in the placenta was low it is possible that the level of MT in the placenta in our experiments was too low to trap increasing amounts of Cd (Figs. 2B, 2C and 2D).

Expression of iso-MT (I, II, and III) genes in the uterus of animals in the 5 mg/kg group was higher than in the other groups. MT-III gene was particularly elevated. However, the level of MT protein did not correspond to the level of MT-III gene expression (Figs. 4A and 4B). Some studies have reported that MT is involved in Cd accumulation and toxicity in genital organs. An increase in Cd accumulation in the placenta resulting from cigarette smoking stimulates expression of placental MT-I and MT-II (Ronco et al., 2005). Accumulation of high levels of Cd in the placenta has been shown to result in trophoblastic damage, which causes to decrease in uteroplacental blood flow, and induce fetal death (Levin et al., 1981). Also, Honda et al. (2010) reported that acute testicular toxicity induced by subcutaneous injection of CdCl\(_2\) is attenuated in MT-III–null mice compared to wild-type mice. Expression of the MT-III gene was observed in the uterus in our study; however, the level of MT protein produced in comparison to expression of MT-III gene suggests that MT-III may not play a role in Cd accumulation in the uterus. Our results suggest that more detailed studies are needed to clarify the role of iso-MTs in the uterus.

The concentration of Cd in the liver and kidney of newborn rats at the fourth week after birth increased with the dose of Cd administered to the mother rat. Particularly, the significant increase of Cd concentration was observed in the kidney of the 5 mg/kg group (Fig. 7). These results suggest that Cd ingested in breast milk is absorbed by the neonatal intestinal tract and bound by intestinal MT. We speculate that Cd is then transferred selectively to the kidney rather than the liver and accumulates in the kidney as MT-Cd.

In newborn rats examined on the first day after birth, the Cd concentration in the liver and kidney increased dependence to the increased dose of Cd administration of the mother rats (Fig.6). Lau et al. (1998) investigated the role of placental MT using genetically altered mice. Their results indicated that MT functions as a placental barrier to Cd transport to the fetus. In our study however, a small amount of Cd had shifted to the fetus. These results suggest that MT does not act as an absolute barrier to Cd transfer, and that a very small amount of Cd passes through the blood-placenta barrier to the fetus. Furthermore, in comparison with Cd distribution of liver and kidney of newborn rats at the fourth week after birth, more Cd was accumulated in liver rather than that in kidney (Figs. 6 and 7). A particularly significant increase in the concentration of Cd was observed in the liver of newborn rats at the first day after birth of the 5 mg/kg group (Fig. 6). In addition, atrophy of placental tissue was observed in mother rats of the 5 mg/kg group (Fig. 5E). From these results, it was considered that non-MT-Cd leaked through the placenta to the fetus as a result of injury to the placental tissue, and accumulated in the fetal liver. Chan et al. (1993) suggested that MT in plasma plays a role in the transport of essential metals such as zinc (Zn) or copper to the fetus. In addition, Carey et al. (2000) demonstrated a relationship between MT and Zn transport from mother rat to fetus with their study using MT-null mice injected with\(^{65}\)Zn. Our results however, suggested that MT does not participate in transport of Cd from the mother to the fetus.

In conclusion, the results of the present study suggested that Cd ingested during the gestation period leaks from the placenta as nonMT-Cd and is taken up by the fetus. Moreover, our results suggested that the iso-MTs MT-I and MT-II act primarily in trapping Cd in the uterus and placenta and thus prevent Cd transport to the fetus. And it was considered that MT has a limited ability to prevent Cd transport from mother rat to fetus.

The participation of metal transporters was also considered as factors on the mechanism of Cd transport into fetus. Recently, It was reported that DMT1 is involved in intestinal absorption of Fe (Gunshin et al., 1997; Ferguson et al., 2001), and the Zip gene family of Zn transporters (Grotz et al., 1998) were shown to participate in the transport of various metals, including Cd. Additional research concerning the role of metal transporters is necessary to
obtain a better understanding of the mechanism whereby Cd is transported between mother and fetus.

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