Pregnancy-Associated Changes in Plasma Metallothionein Concentration and Renal Cadmium Accumulation in Rats (43553)

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Abstract. **Pregnancy-associated changes in metallothionein (MT) concentrations in blood plasma were examined using a competitive enzyme-linked immunosorbent assay with a rabbit polyclonal antibody to rat liver MT. Plasma MT of pregnant rats significantly increased after 8 days of gestation and remained high during pregnancy and for 7 days after delivery. Gel filtration showed that both Cu and Zn were associated with the plasma MT of pregnant rats. These results suggest that plasma MT may play a role in the transport of essential metals such as Cu and Zn to fetus during pregnancy, but the source of plasma MT is unknown. Injections of cadmium chloride and cadmium-metallothionein to pregnant rats further increased the plasma MT concentrations. After injection of $CdCl_2$, both MT and Cd concentrations in the liver of the pregnant rats were significantly lower than those of the nonpregnant rats, whereas renal Cd and MT levels were higher in pregnant rats. This increased accumulation of Cd in the kidney of pregnant rats may be related to the increase of plasma MT during pregnancy. About 5% of 6 pg of *in vitro* added Cd (20 pg/ml) was bound to the MT in the plasma. Therefore, Cd may be transported by the circulating MT to the kidney, leading to an increase in renal accumulation of Cd in pregnant rats.** [P.S.E.B.M. 1993, Vol 202]

Metallothioneins (MT) are low molecular weight metal-binding proteins that can actively sequester intracellular essential metals such as zinc and copper both in placenta and fetal liver during pregnancy, and can regulate their bioavailability (1). Both Zn and Cu are readily transferred from the dams to the embryo through the placenta, but cadmium, a nonessential metal, is not transferred across the placenta, although it is also bound to MT (2). This exposure to Cd during pregnancy results in its accumulation in placenta, and at high levels, can cause teratogenic or embryotoxic effects (3, 4).

Metallothionein levels in rat liver are high during late fetal and early neonatal periods, and MT may play a role in the homeostasis of Zn and Cu (5, 6). The intracellular binding of toxic metals with MT can also provide protection from the toxic effects of certain metals (7). On the contrary, Cd injected parenterally as cadmium-metallothionein (Cd-MT) selectively accumulates in the kidney and leads to proximal tubular damage within hours after the injection at much lower doses than injection of Cd salts (8, 9). An injection of Cd-MT is also teratogenic, but little Cd from this metal-protein complex was detected in the embryo (10). Thus, it has been suggested that Cd teratogenicity may be an indirect effect resulting from renal toxicity or placental haemorrhage.

Pregnancy causes many physiologic changes in the maternal body, and it can also cause tissue redistribution and enhanced toxicity of Cd. Bhattacharyya *et al.* (11, 12) reported that a higher proportion of fed Cd was found in the kidneys of pregnant mice compared to that of nonpregnant mice. It was also reported that rats pregnant for 20 days injected with Cd-MT showed higher nephrotoxicity than nulliparous rats injected with same dose of Cd-MT (13). The reason for these pregnancy-associated changes is not yet understood.

The purpose of the present study was to investigate the changes in MT levels during pregnancy in rats. In addition, relative tissue distribution and toxicity of

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$CdCl_2$ and Cd-MT in pregnant rats and nonpregnant rats were also studied along with MT concentrations in liver, kidney, placenta, and blood plasma after parenteral injections.

Materials and Methods

Preparation of Cd-MT. MT was isolated from livers of male Sprague Dawley rats that had been injected (subcutaneously) with $CdCl_2$ (1 mg Cd/kg) daily for 2 weeks. The purification of isoforms MT-1 and MT-2 was similar to that described previously (14). Briefly, liver samples were homogenized in a 50 m*M* sodium phosphate buffer (pH 7.4), and after centrifugation at 12,000*g* for 10 min at 4°C, the supernatants were heated at 80°C for 2 min. The denatured proteins were removed by centrifugation at 27,000*g* for 10 min (4°C) and the supernatant was applied to a Sephadex G-75 column (5 × 90 cm). Ten thousand molecular weight fractions containing Cd were pooled and lyophilized. The two MT isoforms were separated by polyacrylamide gel electrophoresis in a 7.5% gel under nondenaturing conditions, eluted from the gel, and lyophilized. Only the isoform Cd-MT-2 was used in this study.

Animal Treatments. Virgin female Sprague-Dawley rats (200–250 g) were purchased from Charles River (Montreal, Quebec) and given water and a standard rat chow *ad libitum*. All rats were injected (intraperitoneally) with 40 μg of des-Gly, [D-Ala]-luteinizing hormone-releasing hormone ethylamide (Sigma Chemical Co., St. Louis, MO), a luteinizing hormone to induce ovulation, in 0.2 ml of vehicle solution containing 0.1% bovine serum albumin and 0.9% saline. Five days later, one-half of the rats were bred with male rats of the same strain. Females were checked for vaginal plugs the next morning. Day 1 of gestation was the day on which the vaginal plug occurred. The pregnant rats were separated into three groups. The first group of rats was given six daily injections (subcutaneous) of 1.0 mg Cd/kg body wt as $CdCl_2$ from Days 7 to 12. Rats in the second group were each given two injections (intraperitoneally) of 0.3 mg Cd/kg body wt as Cd-MT on Days 7 and 14. Rats in the third group were each given two injections of 0.9% saline on Days 7 and 14 as controls. An equal number of nonpregnant rats was also separated into three groups and given the same treatments as the pregnant rats. Three rats from each group, both pregnant and nonpregnant, were sacrificed on Days 8, 15, 21, 28, and 35. Rats were sacrificed by exsanguination after pentobarbital (50 mg/kg) anaesthesia. Blood samples were collected in heparinized tubes from abdominal aorta. Liver, kidneys, and placenta (in pregnant rats) were collected and processed for metal and MT analysis. Samples of kidney were fixed in 10% buffered formaldehyde for histopathologic studies.

Chemical Analysis. The Cd content of tissues and plasma was determined by atomic absorption spectroscopy (Varian Spectra-30, Varian Canada, Georgetown, Ontario) after digestion with nitric acid. MT concentrations in tissues and plasma were measured in triplicate by a competitive enzyme-linked immunosorbent assay (ELISA) (15). The ELISA employs the immunoglobulin G fraction of a rabbit antiserum to rat liver MT-2 polymer, which cross-reacts with both MT-1 and MT-2, a biotinylated secondary antibody and peroxidase-conjugated avidin. The detection limit is about 100 pg of MT. Coefficients of variation for reproducibility within and between assays are generally less than 5 and 10%, respectively.

Distribution Profiles of Metals in Blood Plasma. Heparinized blood samples were centrifuged at 1,000*g* for 10 min and the supernatant was taken as plasma samples. An aliquot of plasma sample (0.3 ml) from both pregnant rats (collected at the 16th gestation day) and nonpregnant rats was labeled with 0.5 kBq of ^{109}Cd containing 6 pg of $CdCl_2$. The labeled plasma was fractionated on a calibrated Sephadex G-75 column (0.9 × 60 cm) and the ^{109}Cd radioactivity in the fractions was measured using an LKB 1270 Rackgamma II gamma counter (Pharmacia Inc., Baie d'Urfe, Quebec). A separate aliquot of labeled plasma sample was heated for 5 min at 95°C and centrifuged at 10,000*g* for 10 min to remove the precipitated proteins, and an aliquot (2.5 ml) was lyophilized and reconstituted with H_2O to 0.3 ml before fractionation. Cu and Zn concentrations in the fractions were measured by atomic absorption spectrophotometer (Varian Spectra 30) using air-acetylene flame. The radioactive ^{109}Cd was measured by an LKB 1270 Rackgamma counter with a counting efficiency of 80%.

Statistical Analysis. The data were analyzed by one-way analysis of variance and means of different groups were compared by Student-Newman-Keuls test according to Sokal and Rohlf (16). Five percent was considered as the level of significance.

Results

Concentrations of MT in plasma of nonpregnant control rats (i.e., injected with saline) ranged from 20 to 46 ng ml^{-1}, which can be regarded as the basal level (Fig. 1a) of MT in female rats. Plasma MT levels of pregnant control rats significantly increased after 8 days of gestation. It reached the maximum level (563 ng ml^{-1}) 24 hr before delivery, but returned to normal (nonpregnant) range after the first week of lactation. Nonpregnant rats injected with $CdCl_2$ showed significantly high plasma MT levels on Day 21 (1 week after the last injections) and Day 35 (Fig. 1b). Pregnant rats injected with $CdCl_2$ showed significantly higher plasma MT levels than $CdCl_2$-injected nonpregnant rats on Days 8, 15, and 21 and pregnant control rats on Days 8 and 15. Plasma MT levels of nonpregnant rats injected with Cd-MT increased significantly on Day 8 (24

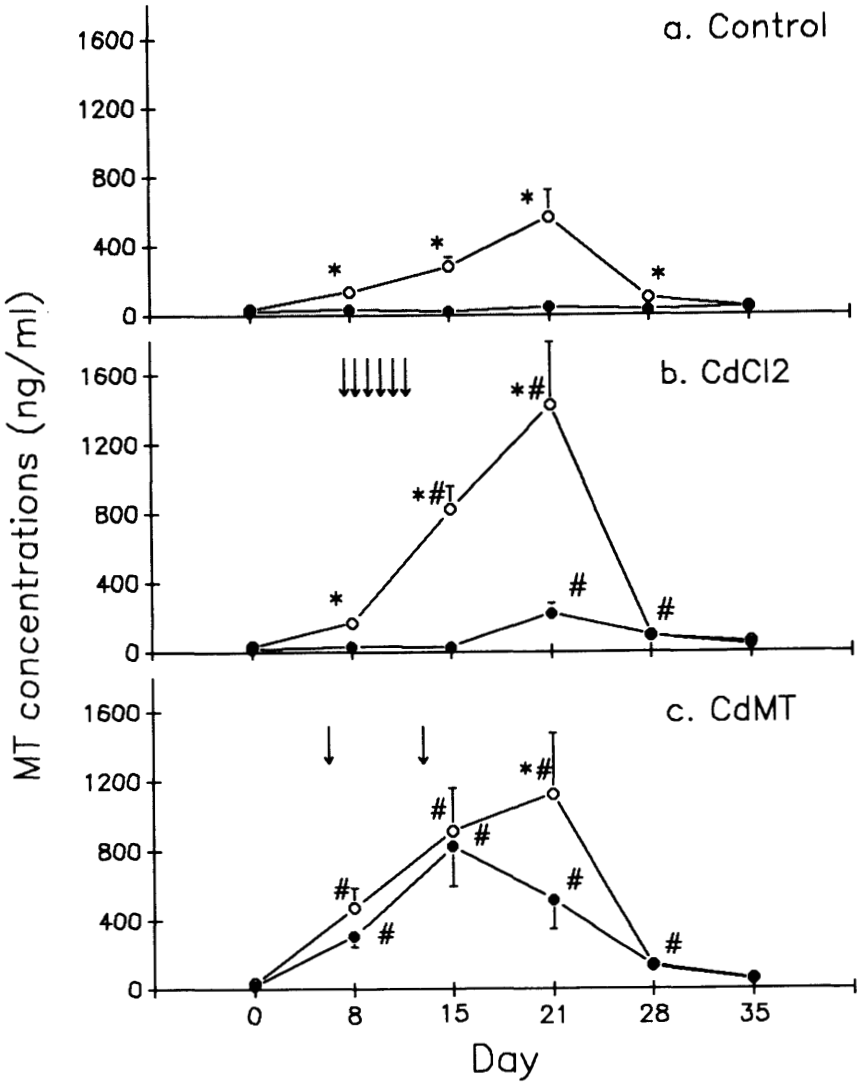

Figure 1. Concentrations of MT in plasma of rats over time. ○, Pregnant rat; ●, nonpregnant rat. Pregnant rats were mated at Day 0 and delivered newborns at Day 21 or 22. (a) Control rats received two injections of 0.3 ml of 0.9% saline on Days 7 and 14, 24 hr before the sacrifice on Days 8 and 15. (b) Rats were given six subcutaneous injections (↓) of 1.0 mg Cd/kg as $CdCl_2$ daily from Days 7 to 12. (c) Rats were given 2 intraperitoneal injections (↓) of 0.3 mg Cd/kg as Cd-MT on Days 7 and 14, 24 hr before the sacrifice on Days 8 and 15. Values represent mean + 1 SD for three animals. *Significantly different from the value of the nonpregnant rats ($P < 0.05$). #Significantly different from the value of the control rats ($P < 0.05$).

hr after the first injection). It reached the maximum on Day 15 (24 hr after the second injections) and remained higher than the basal level until Day 28 (Fig. 1c). There was no significant difference between plasma MT levels of the pregnant and nonpregnant rats injected with Cd-MT on Days 8 and 15. Pregnant rats, however, showed a significantly higher plasma MT level than nonpregnant rats on Day 21.

The concentrations of Cu and Zn in the eluting fractions of plasma samples are shown in Figure 2. Cu and Zn in plasma showed different distributions within the high molecular weight proteins (Fig. 2a). Zn was associated with a higher molecular weight protein (fractions 22–23), which is possibly albumin or globulin (17), compared to that of Cu, which may be ceruloplasmin (fractions 25–27) (18). Similar concentrations of Cu and Zn were found in the fractions corresponding to the MT peak (about 10 kDa, fractions 34–36, $V_e/V_o = 2$). Cu and Zn binding to this small molecular weight protein was not altered by heat treatment of the plasma sample (Fig. 2b).

A typical elution profile of blood plasma labeled *in vitro* with ^{109}Cd is shown in Figure 3a. About 80% of the added Cd in the plasma of nonpregnant rat was associated with high molecular weight proteins (>60 kDa, fractions 20–23). However, only 40% of Cd was found in the corresponding fractions in the plasma of pregnant rats. The other 50% of the added Cd in the plasma was associated with proteins with molecular mass of around 40 kDa (fraction 26–28), which is

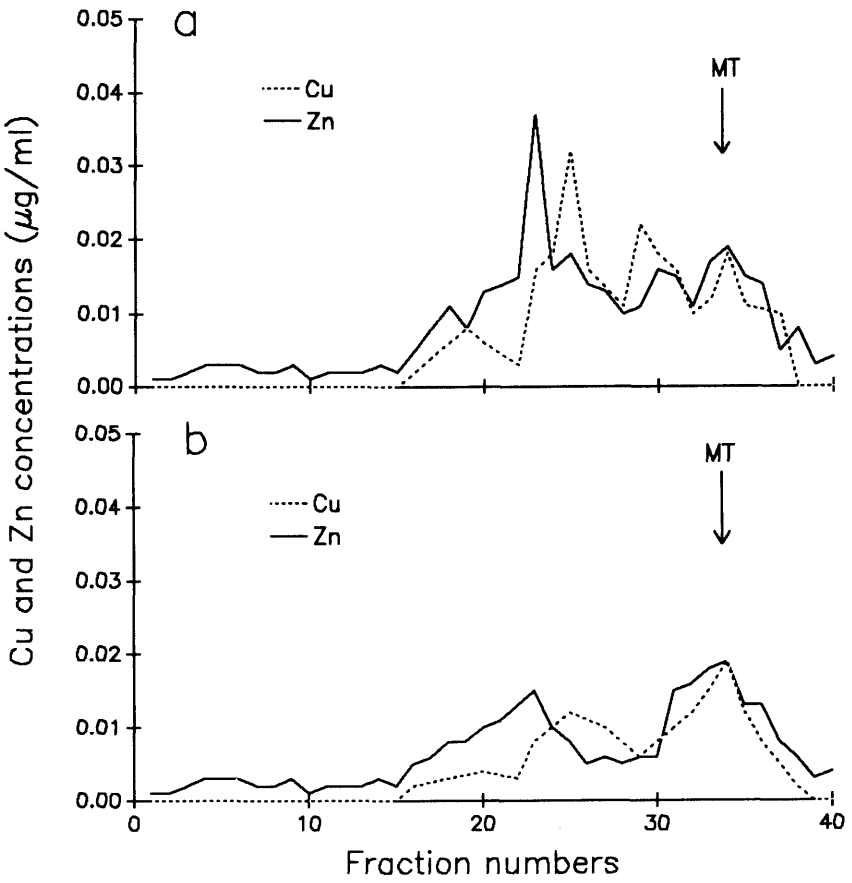

Figure 2. Cu and Zn concentrations in the eluting fractions (0.7 ml) collected from the Sephadex G-75 column (0.9 × 60 cm). Blood plasma (2.5 ml) was obtained from pregnant rats (16th day gestation) and concentrated to 0.3 ml before application to the column. (a) Blood plasma; (b) blood plasma after heating at 95°C for 5 min.

possibly the pregnancy zone protein (19). Moreover, about 5% of the added Cd was associated with the MT elution peak (fractions 34–36). The heat-treated blood plasma sample showed a different elution profile (Fig. 3b). Most of radioactivity associated with the high molecular weight proteins disappeared in plasma of both pregnant and nonpregnant rats. However, the amount of added Cd associated with the MT peak in the pregnant rat plasma remained unchanged.

Concentrations of Cd in liver and kidney of rats injected with $CdCl_2$ were summarized in Table I. Pregnant rats showed a significantly low liver Cd concentration on Days 21 and 28 and a high kidney Cd concentration on Days 28 and 35. Relatively little Cd was found in the liver of both pregnant and nonpregnant rats injected with Cd-MT (Table II). A significantly higher Cd concentration was found in the liver of pregnant rats compared to that of the nonpregnant rat 24 hr after the second injections of Cd-MT.

Concentrations of MT in both liver and kidney of all rats injected with $CdCl_2$ increased with time and reached a maximum on Day 28 (2 weeks after the last injection) (Fig. 4a). MT concentrations in liver were higher than those in kidney of both pregnant and nonpregnant rats. However, liver MT concentrations of pregnant rats were significantly lower than those of nonpregnant rats from Days 15 to 35. Conversely, their kidney MT concentrations were higher than those of nonpregnant rats in the same period of time.

Liver MT concentrations of rats injected with Cd-MT showed only a 2-fold increase (20 $\mu g\ g^{-1}$ c.f. to 10 $\mu g\ g^{-1}$ in control) (Fig. 4b). There was, however, a 10-fold increase in kidney MT concentrations of both pregnant and nonpregnant rats 24 hr after the second injection of Cd-MT. Kidney MT concentrations in pregnant rats were significantly higher than those of nonpregnant rats from Days 21 to 35.

There was a 5-fold increase in the tissue weight of each placenta from gestation Days 15 to 21 (Table III). The total Cd and MT content increased during the same period, even though there was a slight decrease of Cd and MT concentrations. Injection of $CdCl_2$, but not Cd-MT, significantly increased the Cd content in the placenta. However, placenta of rats injected with Cd-MT showed a significantly higher placental MT content at Week 3. Although there was an increase in placenta Cd content after injection of $CdCl_2$, it did not increase the Cd-MT concentrations in the fetuses.

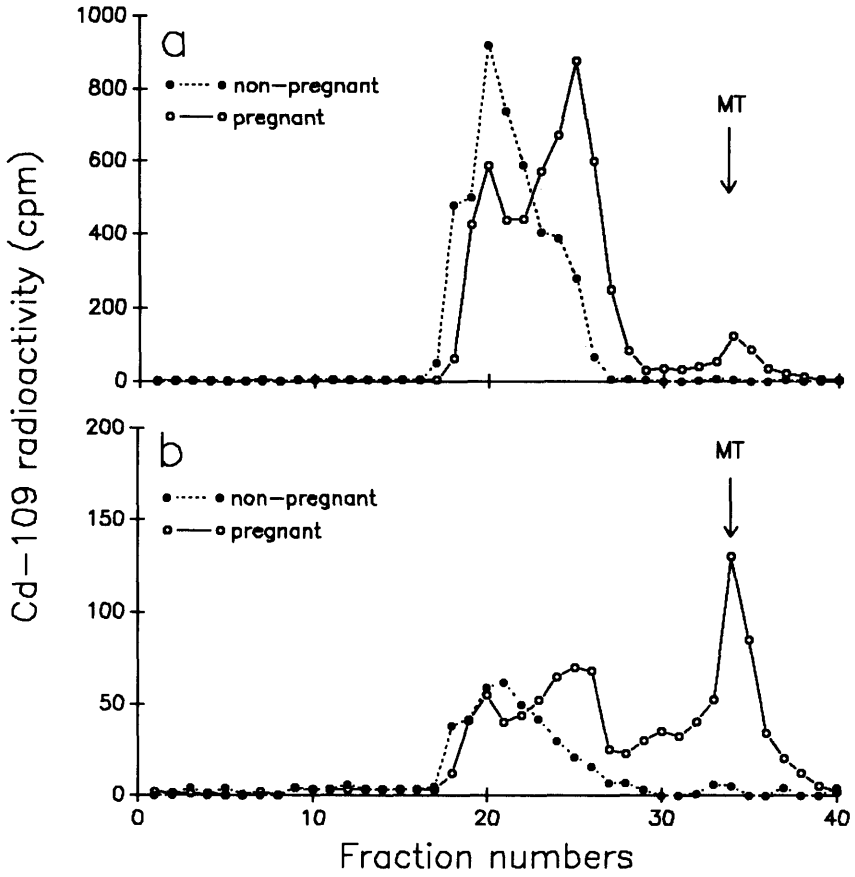

Figure 3. Gel filtration on Sephadex G-75 column (0.9 × 60 cm) of blood plasma sample, obtained from nonpregnant rats and pregnant rats at gestation Day 16 (0.3 ml), labeled with ^{109}Cd *in vitro*. ○, Pregnant rats; ●, nonpregnant rats. (a) Blood plasma; (b) blood plasma after heating at 95°C for 5 min.

Table I. Concentrations of Cd in Liver and Kidney of Pregnant and Nonpregnant Rats Injected with $CdCl_2$

Day	Pregnant rat[a]		Nonpregnant rat[a]	
	Liver	Kidney	Liver	Kidney
0	0.3 ± 0.1[b]	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
8	12 ± 4.2	3.0 ± 1.2	18 ± 6.3	2.4 ± 1.5
15	70 ± 15	34 ± 6.2	88 ± 16	28 ± 7.9
21	46 ± 8.4[c]	50 ± 7.9	91 ± 10	41 ± 11
28	48 ± 12[c]	92 ± 11[d]	88 ± 12	50 ± 8.7
35	70 ± 18	88 ± 14[d]	60 ± 9.5	52 ± 11

[a] Rats were given six subcutaneous injections of 1.0 mg Cd/kg as $CdCl_2$ from Days 7 to 12.
[b] Values are μg/g tissue weight and represent mean ± SD for three animals.
[c] Significantly different from the value in the liver of nonpregnant rats ($P < 0.05$).
[d] Significantly different from the value in the kidney of nonpregnant rats ($P < 0.05$).

Table II. Concentrations of Cd in Liver and Kidney of Pregnant and Nonpregnant Rats Injected with Cd-MT

Day	Pregnant rat[a]		Nonpregnant rat[a]	
	Liver	Kidney	Liver	Kidney
0	0.3 ± 0.1[b]	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
8	1.2 ± 0.4	15 ± 4.7	0.9 ± 0.3	10 ± 4.3
15	5.7 ± 1.8[c]	30 ± 5.2	2.9 ± 1.2	35 ± 8.1
21	2.1 ± 0.6	16 ± 5.5	1.9 ± 0.8	13 ± 6.1
28	1.3 ± 0.5	14 ± 3.9	0.9 ± 0.4	14 ± 4.7
35	1.2 ± 0.9	11 ± 3.6	0.6 ± 0.2	11 ± 3.3

[a] Rats were given two intraperitoneal injections of 0.3 mg Cd/kg as Cd-MT on Days 7 and 14.
[b] Values are μg/g tissue weight and represent mean ± SD for three animals.
[c] Significantly different from the value in the liver of nonpregnant rats ($P < 0.05$).

A progressive necrosis was observed in the convoluted proximal tubules of rats after the first injection of Cd-MT. No difference in morphologic changes was observed between the pregnant rats or nonpregnant rats. None of the $CdCl_2$-injected rats showed any histologic renal damage (data not shown).

Discussion

In the present study, a marked increase in plasma MT containing Zn and Cu during pregnancy has been demonstrated in rats. The basal levels of plasma MT in nonpregnant control female rats in our study were about 30 ng/ml: lower than those reported by Tohyama and Shaikh (20) (around 80 ng/ml), but higher than

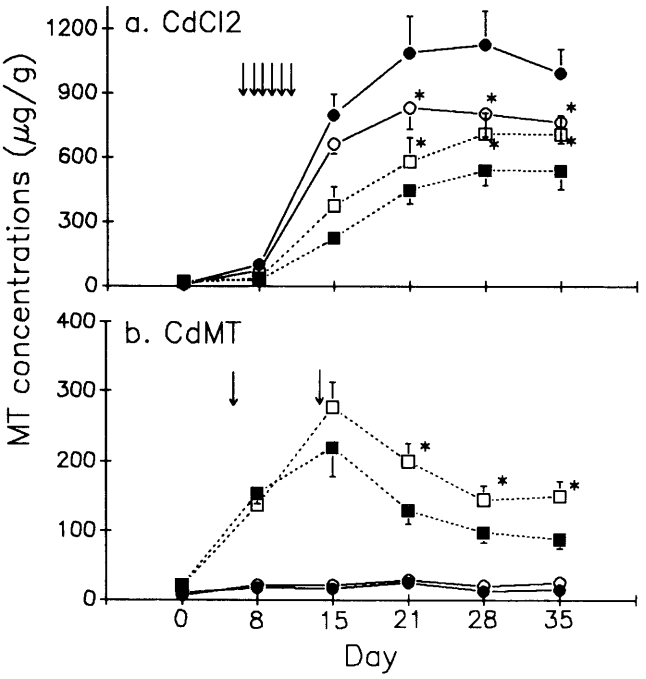

Figure 4. MT concentrations in the liver and kidney of rats over time. ○, Pregnant rat liver; □, pregnant rat kidney; ●, nonpregnant rat liver; ■, nonpregnant rat kidney. (a) Rats were given six subcutaneous injections (↓) of 1.0 mg Cd/kg as $CdCl_2$ daily from Days 7 to 12. (b) Rats were given two intraperitoneal injections (↓) of 0.3 mg Cd/kg as Cd-MT on Days 7 and 15, 24 hr before the sacrifice on Days 8 and 15. Values represent mean + 1 SD for three animals. *Significantly different from the value of nonpregnant rats ($P < 0.05$).

Table III. Cd and MT in Rat Placentae[a]

Treatment[b]	Time	
	Day 15	Day 21
Tissue weight (mg)	120 ± 24	489 ± 55
Cd content (μg)		
Control	0.04 ± 0.01	0.08 ± 0.03
$CdCl_2$	0.19 ± 0.08[c]	0.30 ± 0.16[c]
Cd-MT	0.04 ± 0.02	0.12 ± 0.07
MT content (μg)		
Control	2.8 ± 0.9	6.5 ± 1.4
$CdCl_2$	2.9 ± 1.2	6.0 ± 1.7
Cd-MT	3.2 ± 0.8	8.5 ± 0.5[c]

[a] Values represent mean ± SD for six placentae collected from three rats. Each rat had 14–16 placentae.
[b] Rats were injected with saline (control), $CdCl_2$, and Cd-MT as described in Materials and Methods.
[c] Significantly different from the value of control ($P < 0.05$).

those reported by Mehra and Bremner (21) and Garvey and Chang (22) (2–5 ng/ml). It is difficult to compare the absolute value of MT concentration obtained by immunoassays using different antibodies because of their differences in specificity and sensitivity. The low values reported by Mehra and Bremner (21) may be due to measurement of only MT-1 isoform. However, the relative changes in MT concentrations under different physiologic conditions or after various treatments can be studied and compared after estimation by immunoassay. Plasma MT levels have been shown to be high in newborn rats (23), liver disorders (24), and after exposure to Zn (22), Cd (20, 22), Cu (25), and endotoxin or CCl_4 (26). Increase in plasma MT during pregnancy is a new observation. However, the source of the increased plasma MT during pregnancy in rats is not known. Quaife *et al.* (27) found an increase in MT-1 mRNA in mouse liver during gestation and suggested that hepatic MT synthesis was regulated by glucocorticoid in pregnancy. Piletz *et al.* (28) also reported a 2- to 3-fold increase in the rate of MT synthesis in the liver of pregnant rats compared to those of nulliparous rats. However, no significant change in liver MT concentrations was observed during pregnancy in rats in the present study (data not shown). Because the total MT content in blood plasma was relatively low compared to that in the liver, which is the major reservoir of MT in the body, it is still possible that the increased plasma MT levels in pregnancy may have originated in the liver. Bhattacharyya *et al.* (11) suggested an increase in intestinal MT concentrations during gestation and lactation in mice, but intestinal MT was not measured in their study. Because the total MT content in placentae of pregnant rats increased to a total of about 100 μg (14 placentae each with 6.5 μg) by late gestation, this could be a source of increased plasma MT level. Moreover, the increased plasma MT level during pregnancy may also result from hormonally (e.g., progesterone and cytokine) induced changes in MT in tissues, increase in white blood cell counts in blood, fetus, or general stress of the dams.

Results of the gel filtration chromatography further confirm the presence of MT bound to both Zn and Cu in the plasma of pregnant rats. The elution profile suggests that the MT in the plasma is indeed MT (mol wt about 10,000) and not MT degradation products with retained immunogenicity. Even though a major portion of the plasma Cu and Zn was associated with other blood proteins such as albumin or ceruloplasmin, the increased level of MT suggests that it may also play a role in the transport of these two essential metals to the fetus.

Injection of Cd in the form of $CdCl_2$ or Cd-MT further increased the plasma MT concentration in pregnant rats. Significantly higher Cd concentrations were found in the liver of rats injected with $CdCl_2$ than those injected with Cd-MT. However, Cd-MT-injected rats showed significantly higher Cd accumulation in the kidney than the $CdCl_2$-injected rats. These results were similar to previous reports (7, 8). The most interesting and important difference between $CdCl_2$-injected nonpregnant and pregnant rats is the significantly lower hepatic Cd and MT in pregnant rats. On the other hand, the renal Cd is higher in the $CdCl_2$-injected pregnant rats than nonpregnant rats. This finding is

consistent with the report by Bhattacharyya *et al.* (12), who found a marked effect of parity on accumulation of Cd in kidneys of pregnant mice. However, because the mice were given Cd in drinking water, these findings may be attributed to increased gastrointestinal absorption of Cd. In the present study, rats were injected subcutaneously with $CdCl_2$. The injected Cd is probably bound to the extracellular MT in the plasma in additional to its binding to plasma protein in pregnant rats (29). It has been shown that plasma Cd-MT is freely filtered by the glomerulus and reabsorbed by the proximal convoluted tubules (30, 31), resulting in toxicity to the tubular epithelial cells (32). Even though no obvious sign of necrosis in the proximal tubules of the $CdCl_2$-injected pregnant rats was observed in this study, there may still be an increase in renal dysfunction. Renal tubular dysfunction, particularly glycosuria, has been observed during pregnancy in humans, though this effect is thought to be related to change in renal blood flow (33).

Significantly more Cd was found in the placenta after $CdCl_2$ than Cd-MT injections, whereas higher MT content was found in the placenta of rats injected with Cd-MT. This result suggests that when pregnant rats are injected with Cd-MT, Cd may be transported to the placentae as Cd-MT, but not as "free" Cd, as suggested by Webb *et al.* (10). Moreover, the increased accumulation of Cd in the placenta after $CdCl_2$ injection may be the reason for the previously reported $CdCl_2$-induced placental necrosis (34).

In summary, we report an increase in plasma MT bound to Zn and Cu in rats during pregnancy. Its physiologic significance in the transport of essential metals such as Cu and Zn to the fetus or its toxicologic implication on renal Cd accumulation in pregnancy requires further investigation.

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