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Comparative Cytotoxicity of Exogenous Cadmium-Metallothionein and Cadmium Ion in Cultured Vascular Endothelial Cells

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Cadmium injected as an inorganic salt is transferred to the liver (Maitani and Suzuki 1982) and induces a hepatic injury at high doses (Hoffman et al 1975, Dudley et al 1982). On the other hand, cadmium administered as cadmium-metallothionein (Cd-MT) is selectively transferred to the kidney (Cherian et al 1978, Maitani et al 1984) and damages the organ (Webb and Etienne 1977). Since the major target organ of cadmium is the kidney and the nephrotoxicity is observed even when inorganic cadmium is distributed to the kidneys, Cd-MT leaked from damaged liver cells is suggested to transfer to the kidneys and exhibits the cytotoxicity after the cadmium ions are liberated from the protein (Suzuki et al 1979).

The vascular endothelium is one of the targets of cadmium (Nolan and Shaikh 1986). We have demonstrated that cadmium destroys the monolayer of vascular endothelial cells in a culture system (Kaji et al 1992a). However, the damage of endothelial cells after exposure to Cd-MT has been unclear; this problem is considered to be important for the understanding of cadmium-induced kidney damage as well as vascular lesions. In the present study, the cytotoxicity of Cd-MT was compared with that of cadmium chloride ($CdCl_2$) in cultured vascular endothelial cells.

MATERIALS AND METHODS

Vascular endothelial cells derived from bovine aorta were obtained as a gift from Drs. Yutaka Nakashima and Katsuo Sueishi (First Department of Pathology, Faculty of Medicine, Kyushu University, Fukuoka Japan). Dulbecco's modified Eagle's medium and Hanks' balanced salt solution (HBSS) were purchased from Nissui Pharmaceutical (Tokyo, Japan). ASF 301 medium was from Ajinomoto (Tokyo, Japan). [2,8-^{3}H]Adenine (710.4 GBq/mmol) was obtained from New England Nuclear (Boston, MA, USA). Tissue culture plates and dishes were from Costar (Cambridge, MA, USA). Metallothionein (Cd, Zn-MT) derived from rabbit liver was purchased from Sigma Chemical (St. Louis, MO, USA). PD-10 columns were purchased from Pharmacia AB (Sweden). $CdCl_2$, glutathione (GSH), *L*-cysteine (Cys) and other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

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Metallothionein (5.0 mg) was dissolved in 2.0 ml of 0.1 *M* Tris-HCl buffer solution (pH 8.0) and reacted with 300 μM $CdCl_2$ for 10 min at room temperature. After centrifugation at 3,000 rpm for 10 min, the supernatant was chromatographed on PD-10 column (1.5 × 5 cm) to remove free cadmium ions using 0.1 *M* Tris-HCl buffer solution (pH 8.0) as elution buffer. The concentration of cadmium in the high-molecular-weight fraction (near void volume) was determined by flameless atomic absorption spectrophotometry; Cd-MT without free cadmium ions were confirmed by gel filtration on Superdex 75. This Cd-MT solution was diluted by distilled water and added to the culture medium.

To evaluate the cytotoxicity of $CdCl_2$ and Cd-MT by the [^{3}H]adenine release assay, vascular endothelial cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 24-well culture plates at 37 ℃ in a humid atmosphere of 5% CO_2 in air until confluent (the cell number was approximately 8 × 10^4 cells/cm^2). The medium was changed to fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and the cell layer was metabolically labeled with 37 kBq/ml [^{3}H]adenine for 24 h in the plates. After labelling, the medium was discarded and the cell layer was washed twice with serum-free ASF 301 medium. The cell layer was then incubated at 37 ℃ for 24 h in 0.25 ml of serum-free ASF 301 medium in the presence of $CdCl_2$ or Cd-MT at 2.0 or 5.0 μM as cadmium. In another experiment, the cell layer was washed twice with HBSS and incubated at 37 ℃ for 6 hrs in 0.25 ml of HBSS with $CdCl_2$ at 50 or 100 μM in the presence or absence of GSH or Cys at 0.2, 0.5 or 1.0 mM. After incubation, the medium or HBSS was collected and the cell layer was washed with 0.25 ml of CMF-PBS; the wash was combined with the corresponding medium or HBSS. The cell lysate was prepared by addition of 0.25 ml of 0.5% sodium dodecyl sulfate to the cell layer; after collecting the cell lysate, the cultured well was washed with 0.25 ml of CMF-PBS and the wash was combined with the corresponding cell lysate. The radioactivity either in the cell layer or in the medium or HBSS was measured by liquid scintillation counting. The [^{3}H]adenine release (%) was calculated by dividing the radioactivity (dpm) in the medium (or HBSS) by that (dpm) found in both the medium (or HBSS) and the cell lysate.

The accumulation of intracellular cadmium was determined as follows : Vascular endothelial cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 6-well culture plates until confluent. After culture, the medium was changed to 1.0 ml of fresh serum-free ASF 301 medium and incubated at 37 ℃ for 24 h in the presence of $CdCl_2$ or Cd-MT at 2.0 or 5.0 μM as cadmium. In another experiment, confluent cultures of endothelial cells were treated with $CdCl_2$ at 50 or 100 μM combined with or without GSH or Cys at 0.2, 0.5 or 1.0 m*M* for 6 h in 1.0 ml of HBSS. The treated cell layer was washed twice with CMF-PBS and re-washed three times with 0.75 ml of CMF-PBS containing 2 m*M* EGTA at 4 ℃ to remove cadmium loosely bound to the cell surface (Blazka and Shaikh 1991). The cell layer was then extracted with 1.0 ml of 0.1 *M* acetate buffer solution (pH 5.5) containing 10 m*M* EDTA at 4 ℃ for 72 h. The extract was collected and the cell layer was washed with 1.0 ml of the buffer; the wash was com-

bined with the etract. The content of cadmium in the extract was measured by flameless atomic absorption spectrophotometry. The cell layer after extraction was scraped off with a rubber policeman in the presence of 0.75 ml of CMF-PBS; the culture well was washed with 0.75 ml of CMF-PBS and the wash was combined with the cell suspension. The cell homogenate was prepared by sonication and analyzed for the DNA content by the fluorometric method (Kissane and Robins 1958).

Confluent cultures of endothelial cells in 60-mm dishes were treated with $CdCl_2$ or Cd-MT at 2.0 or 5.0 μM for 24 h in serum-free ASF 301 medium. After treatment, the cell layer was scraped off with a rubber policeman in the presence of 1.0 ml of 0.25 *M* sucrose. The dish was washed with 1.0 ml of 0.25 *M* sucrose and the wash was combined with the harvested cell suspension. The cell homogenate was prepared by sonication and analyzed for the metallothionein content by the method of Onosaka et al (1978) with some modifications. An aliquot of the cell homogenate was used for the assay of DNA content by the fluorometric method.

Data were analyzed for statistical significance by Student's *t*-test. *P* values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS AND DISCUSSION

Firstly, the cytotoxic effect of $CdCl_2$ and Cd-MT on cultured vascular endothelial cells was compared. As shown in Table 1, $CdCl_2$ markedly augmented the [^{3}H]adenine release, suggesting that the cadmium ions are strong cytotoxic. However, Cd-MT did not show such a cytotoxic effect. These results clearly indicated that not only intracellular but also extracellular metallothionein plays a protective role against cadmium cytotoxicity in vascular endothelial cells. In other words,

Table 1. The cytotoxicity of cadmium chloride ($CdCl_2$) and cadmium metallothionein (Cd-MT) and the accumulation of intracellular cadmium and metallothionein in cultured vascular endothelial cells.

	[^{3}H]Adenine release (%)	Cd content (nmol/μg DNA)	MT content (ng/μg DNA)
Control	19.40 ± 0.12	-	15.65 ± 2.10
2.0 μM $CdCl_2$	29.98 ± 0.72	0.138 ± 0.012	52.56 ± 4.56
2.0 μM Cd-MT	21.13 ± 0.93***	0.058 ± 0.003**	51.96 ± 4.24
5.0 μM $CdCl_2$	37.66 ± 0.35	0.254 ± 0.015	53.33 ± 5.90
5.0 μM Cd-MT	21.76 ± 0.56***	0.069 ± 0.003**	54.34 ± 5.90

Confluent cultures of bovine aortic endothelial cells were incubated at 37 °C for 24 hrs in serum-free ASF 301 medium in the presence of $CdCl_2$ or Cd-MT at 2.0 or 5.0 μM as cadmium. Values are means ± SE of five samples. Significantly different from the corresponding $CdCl_2$ group, **P<0.01; ***P<0.001.

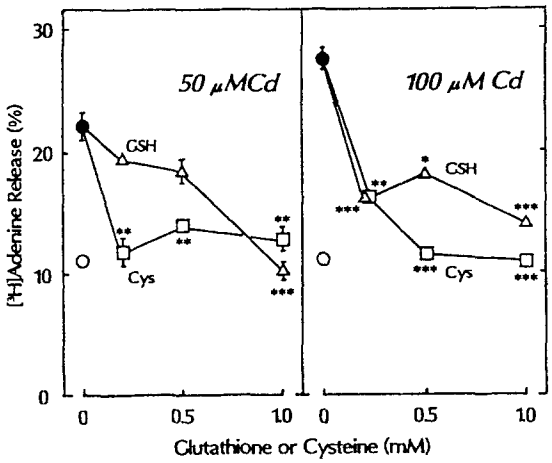

Figure 1. Effect of glutathione (GSH) and cysteine (Cys) on cadmium-induced increase in the [^{3}H]adenine release from cultured vascular endothelial cells. Confluent cultures of bovine aortic endothelial cells were incubated at 37 °C for 6 hrs in HBSS with $CdCl_2$ at 50 μM (left panel) or 100 μM (right panel) in the presence or absence of GSH or Cys at 0.2, 0.5 or 1.0 mM each. Values are means ± SE of four samples. Significantly different from the corresponding $CdCl_2$ treatment, $*P<0.05$; $**P<0.01$; $***P<0.001$. ○, control; ●, $CdCl_2$ treatment; △, $CdCl_2$ plus GSH treatment; □, $CdCl_2$ plus Cys treatment.

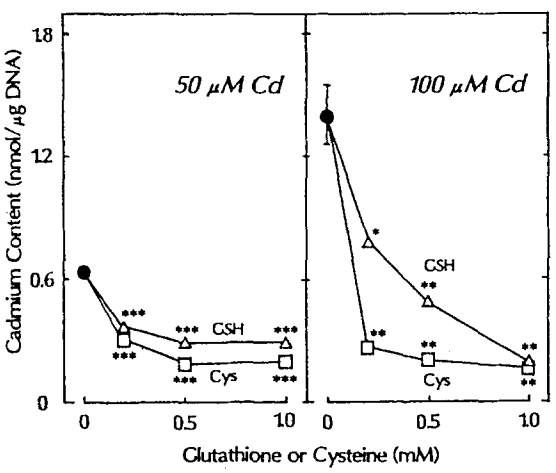

Figure 2. Effect of glutathione (GSH) and cysteine (Cys) on the accumulation of cadmium in cultured vascular endothelial cells. Confluent cultures of bovine aortic endothelial cells were incubated at 37 °C for 6 hrs in HBSS with $CdCl_2$ at 50 μM (left panel) or 100 μM (right panel) in the presence or absence of GSH or Cys at 0.2, 0.5 or 1.0 mM each. Values are means ± SE of four samples. Significantly different from the corresponding $CdCl_2$ treatment, $*P<0.05$; $**P<0.01$; $***P<0.001$. ●, $CdCl_2$ treatment; △, $CdCl_2$ plus GSH treatment; □, $CdCl_2$ plus Cys treatment.

extracellular cadmium bound to metallothionein was only a slightly cytotoxic to endothelial cells.

The accumulation of intracellular cadmium in endothelial cells was significantly less in the Cd-MT groups than in the corresponding $CdCl_2$ groups (Table 1), suggesting that the protection by extracellular metallothionein against cadmium cytotoxicity may be primarily due to a reduction of cadmium uptake rather than an enhancement of metallothinein synthesis by the cells. Similar level of intracellular metallothionein (Table 1) supports this postulation.

Figure 1 shows the effect of GSH and Cys on the cytotoxicity of $CdCl_2$. To avoid the influence of Cys in the medium, endothelial cells were treated with $CdCl_2$ in HBSS. The increase in [^{3}H]adenine release induced by $CdCl_2$ was significantly diminished by either GSH or Cys. At that time, either GSH or Cys reduced the intracellular cadmium content in a dose-dependent manner (Fig. 2). These results clearly indicated that cysteine residues may be involved in the protection by extracellular metallothionein against cadmium cytotoxicity. Similar results were observed in porcine kidney epithelial LLC-PK1 cells (Bruggeman et al 1992).

We speculate that cadmium bound to ligands such as metallothionein, GSH and Cys has little or no ability to enter endothelial cells. It is possible that liberation of cadmium from the ligands may be essential for entrance of the metal into the cells. When cadmium ions entered, they would induce the synthesis of metallothionein (Kaji et al 1993) and would be bound to the newly synthesized metallothionein (Kaji et al 1992b), although a possibilty that intact Cd-MT was taken up by the cells is excluded.

In conclusion, it was found that cadmium bound to metallothionein accumulates less within cultured vascular endothelial cells and exhibits lower cytotoxicity. It is thus likely that cadmium-induced functional damage of endothelial cells including inhibition of the proliferation (Kaji et al 1992a) and stimulation of plasminogen activator inhibitor type 1 release (Yamamoto et al 1993) also would be less in Cd-MT-exposed cells. The present data support the hypothesis that the toxicity of Cd-MT and Cd-Cys (Maitani et al 1986) in blood *in vivo* is due to the cadmium ions liberated from metallothionein and other ligands from endothelial cell level.

REFERENCES

Blazka ME, Shaikh ZA (1991) Differences in cadmium and mercury uptakes by hepatocytes: Role of calcium channels. Toxicol Appl Pharmacol 110 : 355-363

Bruggeman IM, Temmink JHM, van Bladeren PJ (1992) Effect of glutathione and cysteine on apical and basolateral uptake and toxicity of $CdCl_2$ in kidney cells (LLC-PK_1). Toxic in Vitro 6 : 195-200

Cherian MG, Goyer RA, Valberg LS (1978) Gastrointestinal absorption and organ distribution of oral cadmium chloride and cadmium-metallothionein in mice. J Toxicol Environ Health 4 : 861-868

Dudley RE, Svoboda DJ, Klaassen CD (1982) Acute exposure to cadmium

causes severe liver injury in rats. Toxicol Appl Pharmacol 65 : 302-313
Hoffman EO, Cook JA, DiLuzio NR, Coover JA (1975) The effects of acute cadmium administration in the liver and kidney of the rat. Lab Invest 32 : 655-664
Kaji T, Mishima A, Yamamoto C, Sakamoto M, Koizumi F (1992a) Effect of cadmium on the monolayer maintenance of vascular endothelial cells in culture. Toxicology 71 : 267-276
Kaji T, Mishima A, Koyanagi E, Yamamoto C, Sakamoto M, Kozuka H (1992b) Possible mechanism for zinc protection against cadmium cytotoxicity in cultured vascular endothelial cells. Toxicology 76 : 257-270
Kaji T, Yamamoto C, Tsubaki S, Ohkawara S, Sakamoto M, Sato M, Kozuka H (1993) Metallothionein induction by cadmium, cytokines, thrombin and endothelin-1 in cultured vascular endothelial cells. Life Sci 53 : 1185-1191
Kissane JM, Robins E (1958) The fluorometric measurement of deoxyribonucleic acid in animal tissue with special reference to the central nervous system. J Biol Chem 233 : 184-188
Maitani T, Suzuki KT (1982) Changes of essential metal levels in selected tissues and splenomegaly induced by the injection of suspending cadmium salt into mice. Toxicol Appl Pharmacol 62 : 219-227
Maitani T, Waalkes MP, Klaassen CD (1984) Distribution of cadmium after oral administration of cadmium-thionein to mice. Toxicol Appl Pharmacol 74 : 237-243
Maitani T, Watahiki A, Suzuki KT (1986) Acute renal dysfunction by cadmium injected with cysteine in relation to renal critical concentration of cadmium. Arch Toxicol 58 : 136-140
Nolan CV, Shaikh ZA (1986) The vascular endothelium as a target tissue in acute cadmium toxicity. Life Sci 39 : 1403-1409
Onosaka S, Tanaka K, Doi M, Okahara K (1981) A simplified procedure for determination of metallothionein in animal tissue. Eisei Kagaku 24 : 128-131
Suzuki KT, Maitani T, Takenaka S (1979) Fate of intraperitoneally injected liver metallothionein in rat kidney. Chem Pharm Bull 27 : 647-653
Webb M, Etienne AT (1977) Studies on the toxicity and metabolism of cadmium-thionein. Biochem Pharmacol 26 : 25-30
Yamamoto C, Kaji T, Sakamoto M, Kozuka H (1993) Cadmium stimulation of plasminogen activator inhibitor-1 release from human vascular endothelial cells in culture. Toxicology 83 : 215-223