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SHORT COMMUNICATION

Masahiko Satoh · Yasunobu Aoki · Chiharu Tohyama

Protective role of metallothionein in renal toxicity of cisplatinum

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Abstract To elucidate the protective role of metallothionein (MT) in the prevention of cisplatinum (*cis*-DDP) toxicity, we investigated the lethal and renal toxicities caused by *cis*-DDP in MT-null transgenic mice in comparison with wild-type control mice, and examined the effect of pretreatment with bismuth nitrate or zinc sulfate on the *cis*-DDP nephrotoxicity. The MT-null mice were of mixed 129 Ola and C57BL/6 genetic background. Since no differences in *cis*-DDP nephrotoxicity were observed between these strains, C57BL/6J mice were used as the wild-type control. The basal MT levels in the kidneys were negligible in the MT-null mice and 2.93 ± 0.77 μg/g tissue in the C57BL/6J mice. In terms of both the lethal and renal toxicities of *cis*-DDP, MT-null mice were far more sensitive than C57BL/6J mice. Preinduction of renal MT synthesis by administration of bismuth nitrate or zinc sulfate protected C57BL/6J mice from *cis*-DDP nephrotoxicity. In the case of MT-null mice, however, renal MT could not be induced by pretreatment with these metal compounds, and renal toxicity of *cis*-DDP was not prevented by this pretreatment. These results suggest that MT is an important factor with the potential to suppress the development of *cis*-DDP toxicity.

Key words Cisplatinum · Metallothionein · Bismuth · Zinc · Renal toxicity

Introduction

Cisplatinum (*cis*-DDP), a coordination complex of platinum, is the most extensively evaluated antineoplastic agent developed thus far. It has potent antitumor activity and a broad spectrum of anticancer activity in humans [16, 21]. However, *cis*-DDP produces severe side effects such as renal, bone marrow and gastrointestinal toxicities [13]. In particular, the clinical use of *cis*-DDP is limited by its renal toxicity. It has been reported that preadministration of metals that induce metallothionein (MT) such as bismuth and zinc can prevent the toxic side effects of *cis*-DDP [18, 25]. MT is a cysteine-rich low molecular weight protein with a high affinity for metals such as zinc, copper, cadmium, mercury and platinum, and is found ubiquitously in tissues of many animal species [8]. MT is induced by various metals, glucocorticoids and many other factors and is considered to play a role in the homeostasis of essential metals such as zinc and copper [8]. Elevated cellular MT levels have been shown to protect the cells against the toxicities of heavy metals, mutagens, anticancer agents and radical-inducing substances [8].

Recent studies have shown that the administration of bismuth compounds prior to the injection of *cis*-DDP reduces the lethal and renal toxicities of this drug in mice without compromising its antitumor activity [10, 19, 23]. Moreover, some clinical trials have suggested that pretreatment with bismuth compounds is effective in reducing the renal toxicity of *cis*-DDP [27, 28]. Naganuma et al. [19] speculated that this protective effect of bismuth pretreatment against *cis*-DDP toxicity is exerted by selective induction of MT by bismuth in the kidney, a major target organ of *cis*-DDP toxicity, but not in the tumor tissues. On the other hand, pretreatment with zinc compounds reduces not only the renal toxicity of *cis*-DDP but also its antitumor activity, while, as expected, the MT content in the tumor as well as the kidneys is increased after treatment with zinc [24]. However, it is still unclear whether MT acts as a major protecting factor against *cis*-DDP toxicity. The role of endogenous MT in *cis*-DDP toxicity has not been studied.

Recently, transgenic mice deficient in the MT-I and MT-II genes (MT-null mice) have been established

M. Satoh (✉) · Y. Aoki · C. Tohyama
Environmental Health Sciences Division,
National Institute for Environmental Studies,
16-2 Onogawa, Tsukuba,
Ibaraki 305, Japan
Tel. +81-298-50-2448; Fax +81-298-50-2588;
E-mail: masahiko@nies.go.jp

[14, 17] and are thought to be a good model to study the protective role of MT against *cis*-DDP toxicity. In the present study, we examined sensitivity of MT-null transgenic mice to the lethal and renal toxicities of *cis*-DDP and also investigated the effect of pretreatment with bismuth and zinc on the renal toxicity of *cis*-DDP.

Materials and methods

Animals and chemicals

MT-null mice whose MT-I and II genes have a null mutation were produced and kindly provided by Dr. A. Choo (Murdoch Institute for Research into Birth Defects, Royal Children's Hospital, Australia) [17] and were routinely bled in the vivarium of the National Institute for Environmental Studies (NIES). Microbiological and viral examinations were performed for different colonies over a 1-year period as well as regular quarantine procedures, and neither pathogenic infections nor significant phenotypical abnormalities were found. The MT-null mice were of mixed 129 Ola and C57BL/6 genetic background. As wild-type control mice, 8-week-old female C57BL/6J and 129/Sv mice were purchased from Japan Clea Co. (Tokyo, Japan). *cis*-DDP was supplied by Nippon Kayaku Co., Tokyo, Japan. Metal compounds and other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Treatments

Female MT-null, C57BL/6J and 129/Sv mice were randomized at 9 weeks of age into control and experimental groups. C57BL/6J and 129/Sv mice were injected intraperitoneally (i.p.) with *cis*-DDP (30, 40 and 50 μmol/kg). MT-null and C57BL/6J mice were given i.p. injections of *cis*-DDP at doses between 20 and 60 μmol/kg. To examine the renal toxicity of *cis*-DDP (four mice for each dose group), blood was collected from each mouse under diethylether anesthesia 4 days after injection. The survival rate of these mice (seven mice for each dose group) was determined 20 days after injection of *cis*-DDP (30, 40, 50 and 60 μmol/kg).

On day 0, mice (four mice for each dose group) were given subcutaneous (s.c.) injections of $Bi(NO_3)_3$ (50 μmol/kg), $ZnSO_4$ (100 μmol/kg) or saline once a day for 2 days. $Bi(NO_3)_3$- $ZnSO_4$- or saline-injected mice were injected i.p. with *cis*-DDP (30 and 40 μmol/kg) on day 2 (24 h after the last injection of metal compounds). Some of these treated mice were sacrificed by cervical dislocation to determine the renal MT level on day 2 (at the time of *cis*-DDP injection). On day 6 (4 days after the *cis*-DDP injection), blood was collected from each mouse under diethylether anesthesia to evaluate the renal toxicity of *cis*-DDP. Mice were handled with humane care throughout this study according to the NIES guidelines.

Histochemical staining

For histochemical evaluation of nephrotoxicity, kidney tissues were fixed in 10% buffered formalin and embedded in paraffin. Deparaffinized tissue sections of 5 μm thickness were stained with hematoxylin-eosin.

Analysis

Blood urea nitrogen (BUN) and plasma creatinine values were determined using an automatic dry-chemistry analyzer system (Spotchem SP-4410; Kyoto Daiichikagaku, Kyoto, Japan). The MT content in the kidneys was measured by radioimmunoassay [20]. In order to estimate glutathione (GSH) levels, renal nonprotein SH (NP-SH) level was determined by the method of Ellman [5] using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) as described by Costa and Murphy [4]. The data were analyzed using Student's *t*-test.

Results

The basal MT levels in the kidneys of C57BL/6J, 129/Sv and MT-null mice were 2.93 ± 0.77 μg/g tissue, 3.07 ± 0.67 μg/g tissue and below the limit of detection (< 0.2 μg/g tissue), respectively. The renal NP-SH levels in these strains were similar to one another: C57BL/6J mice, 3.03 ± 0.38 μmol/g tissue; 129/Sv mice, 3.15 ± 0.41 μmol/g tissue; MT-null mice, 3.31 ± 0.47 μmol/g tissue. The renal toxicity of *cis*-DDP in MT-null, C57BL/6J and 129/Sv mice was evaluated in terms of the BUN and plasma creatinine values. C57BL/6J and 129/Sv mice responded in a very similar manner to *cis*-DDP in terms of BUN and creatinine values (data not shown). Thus, in the following experiments, C57BL/6J mice were used as the wild-type control.

As shown in Fig. 1, BUN and creatinine values of *cis*-DDP-treated C57BL/6J mice were significantly increased in a dose-dependent manner above a *cis*-DDP dose of 40 μmol/kg, whereas these values were at control levels with *cis*-DDP doses of 20 and 30 μmol/kg. In the MT-null mice, BUN and creatinine values were significantly

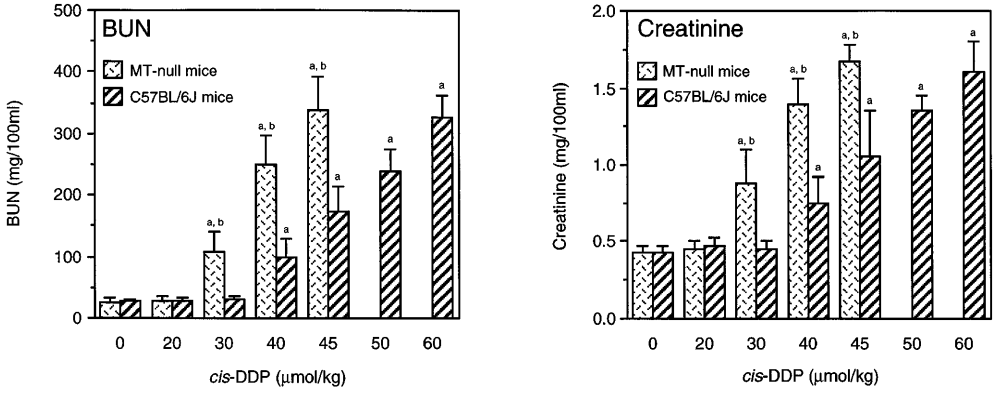

Fig. 1 Renal toxicity of *cis*-DDP in MT-null mice and C57BL/6J mice. Mice were injected i.p. with *cis*-DDP (20–60 μmol/kg). Renal toxicity was determined 4 days after the *cis*-DDP injection. The values are means ± SD from four mice *a* significantly different from corresponding groups, $P < 0.01$; *b* significantly different from C57BL/6J mice, $P < 0.01$

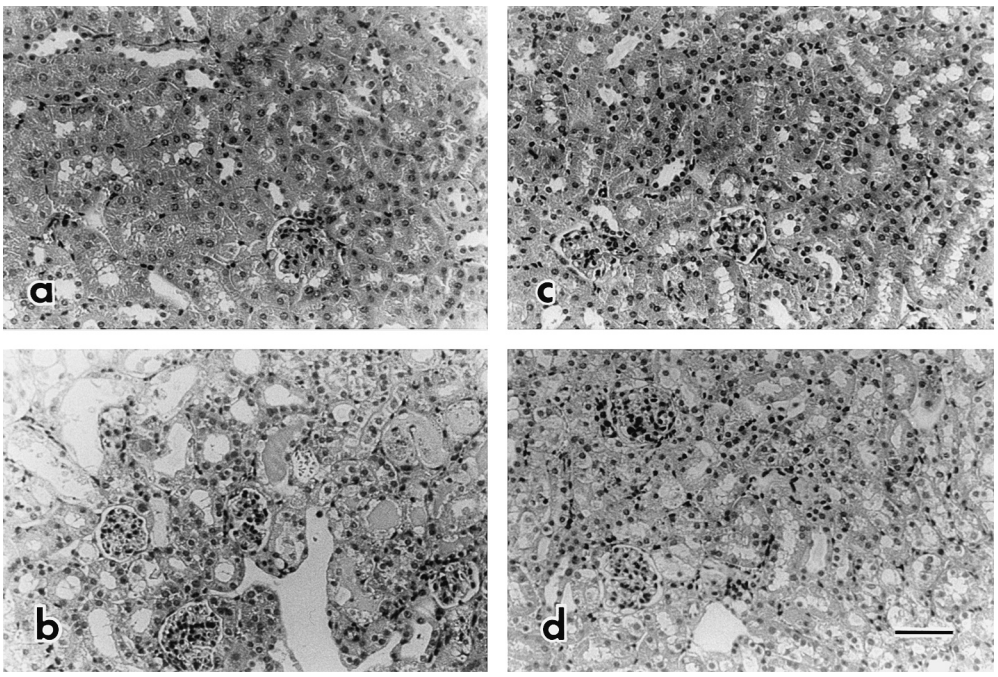

Fig. 2a–d Histopathological changes in the renal cortex of MT-null mice and C57BL/6J mice treated with *cis*-DDP. Mice were injected i.p. with *cis*-DDP (40 μmol/kg). Histopathological changes in the kidney were evaluated 4 days after the *cis*-DDP injection. The kidney was lightly stained with hematoxylin-eosin (**a** untreated MT-null mice, **b** 40 μmol/kg *cis*-DDP -treated MT-null mice, **c** untreated C57BL/6J mice, **d** 40 μmol/kg *cis*-DDP -treated C57BL/6J mice; *bar* 50 μm)

increased above a *cis*-DDP dose of 30 μmol/kg, and these increases were also dependent on the *cis*-DDP dose. MT-null mice injected with 50 and 60 μmol/kg of *cis*-DDP died within 4 days of injection. Furthermore, BUN and creatinine values of *cis*-DDP-injected MT-null mice were markedly increased compared with those of C57BL/6J mice at doses of 30, 40 and 45 μmol/kg. Histopathological examination confirmed this enhancement of the kidney damage in MT-null mice caused by *cis*-DDP (Fig. 2). In MT-null mice, marked morphological changes such as degeneration and necrosis in the proximal tubular cells and a dilated tubular lumen were observed following *cis*-DDP injection at a dose of 40 μmol/kg (Fig. 2b). In contrast, the C57BL/6J mice treated with the same *cis*-DDP dose showed a lesser degree of tubular damage (Fig. 2d).

The lethal toxicity of *cis*-DDP was examined in the MT-null mice and C57BL/6J mice. The survival rates of C57BL/6J mice injected with various doses of *cis*-DDP were: 30 μmol/kg, 100%; 40 μmol/kg, 100%; 50 μmol/kg, 57%; and 60 μmol/kg, 0%. The equivalent survival rates for MT-null mice were: 30 μmol/kg, 100%; 40 μmol/kg, 57%; 50 μmol/kg, 0% and 60 μmol/kg, 0%. Since the LD_{50} values of *cis*-DDP in MT-null and C57BL/6J mice were approximately 45 and 55 μmol/kg and *cis*-DDP induced death in both types of mice in a dose-dependent manner, it is clear that MT-null mice were more sensitive than C57BL/6J mice to *cis*-DDP toxicity.

The renal MT levels were significantly higher in C57BL/6J mice treated with $Bi(NO_3)_3$ and $ZnSO_4$ than in untreated mice (Table 1). However, the amount of renal MT was negligible in untreated MT-null mice and could not be induced by $Bi(NO_3)_3$ or $ZnSO_4$. On the other hand, the renal NP-SH levels in both the MT-null and C57BL/6J mice were not affected by injection of either compound. Figure 3 shows the effects of pretreatment with $Bi(NO_3)_3$ and $ZnSO_4$ on the renal toxicity of *cis*-DDP in MT-null mice and C57BL/6J mice. BUN and creatinine values in the C57BL/6J mice were significantly increased by the injection of *cis*-DDP at a dose of 40 μmol/kg but not at 30 μmol/kg. Pretreatment with $Bi(NO_3)_3$ or $ZnSO_4$ clearly cancelled these effects of *cis*-DDP on BUN and creatinine values. In contrast, pretreatment of MT-null mice with either compound did not affect the elevated levels of BUN and creatinine caused by *cis*-DDP at doses of 30 and 40 μmol/kg.

Table 1 MT and NP-SH contents in the kidneys of MT-null mice and C57BL/6J mice treated with metal compounds. Mice were injected s.c. with $Bi(NO_3)_3$ (50 μmol/kg) or $ZnSO_4$ (100 μmol/kg) once a day for 2 days. Renal MT and NP-SH contents were determined 24 h after the last injection of each metal compound. The values are mean ± SD for four mice. $^*P < 0.001$ vs control

	MT content (μg/g tissue)		NP-SH content (μmol/g tissue)	
	C57BL/6J	MT-null	C57BL/6J	MT-null
Control	3.15 ± 1.10	< 0.2	3.00 ± 0.54	3.12 ± 0.60
$Bi(NO_3)_3$	78.60 ± 24.98*	< 0.2	3.14 ± 0.61	3.32 ± 0.73
$ZnSO_4$	106.50 ± 17.35*	< 0.2	2.75 ± 0.47	3.26 ± 0.51

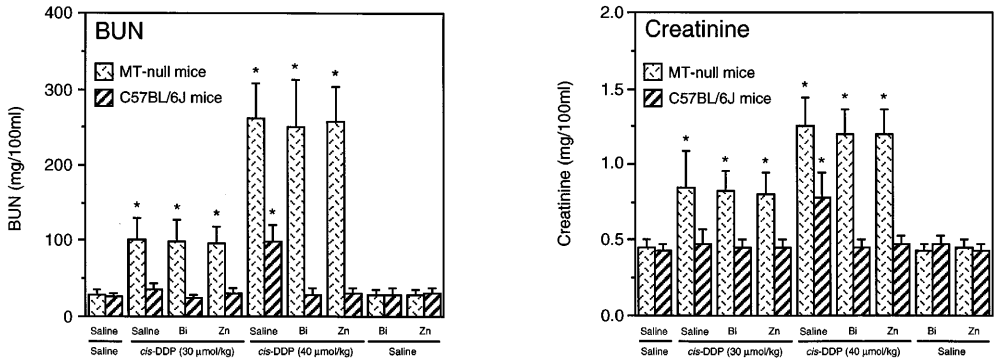

Fig. 3 Effect of pretreatment with metal compounds on renal toxicity of *cis*-DDP in MT-null mice and C57BL/6J mice. Mice were given s.c. injections of $Bi(NO_3)_3$ (50 μmol/kg) or $ZnSO_4$ (100 μmol/kg) once a day for 2 days. $Bi(NO_3)_3$- and $ZnSO_4$-injected mice were injected i.p. with *cis*-DDP (30 and 40 μmol/kg) 24 h after the last injection of each metal compound. Renal toxicity was determined 4 days after the *cis*-DDP injection. The values are means ± SD from four mice. $^*P < 0.01$ vs corresponding group

Discussion

Several cell culture studies have shown that overexpressed MT prevents the cytotoxicity caused by *cis*-DDP [2]. In addition, tumor cells that acquire resistance to *cis*-DDP frequently have increased levels of MT and MT mRNA [9], and the reversal of the *cis*-DDP resistance phenotype is accompanied by a decrease in MT content [9]. Kondo et al. [11] have recently reported that fibroblasts derived from MT-null mouse embryos are more sensitive to *cis*-DDP than MT-positive normal fibroblasts. These in vitro studies suggest that cellular MT levels determine the sensitivity of mammalian cells to *cis*-DDP. The present in vivo studies support this notion and showed that endogenous MT and induced MT in the kidney protect mice against the lethal and renal toxicities of *cis*-DDP, because the sensitivity to the toxicities was also increased in the MT-null mice.

Since the MT-null mice used in this study were of mixed 129 Ola and C57BL/6 genetic background, we sought to determine whether there were strain differences in the renal toxicity caused by *cis*-DDP, but found that the two strains, C57BL/6J and 129/Sv, responded in a very similar manner to *cis*-DDP. Moreover, the basal MT level was similar in the two strains. GSH plays an important role in the detoxification of *cis*-DDP [1, 15, 30], and we found that the basal NP-SH level was also similar among MT-null, C57BL/6J and 129/Sv mice. Therefore, the MT-null strain is a good model to study the role of MT in *cis*-DDP nephrotoxicity.

In previous studies we have demonstrated that adverse effects of *cis*-DDP are prevented by preadministration of bismuth compounds [10, 19, 23]. In addition, the significant correlation observed between the protective effect and the preinduced MT level in the kidney suggested the participation of renal MT increased by bismuth in the protection of the tissue against *cis*-DDP toxicity. This possibility was also supported by the fact that the protective effect of bismuth against *cis*-DDP toxicity occurred only when the bismuth was administered prior to *cis*-DDP. Thus, this protective effect of metals against *cis*-DDP toxicity is thought to result from the preinduction of MT synthesis in the target organ [10, 19]. However, it was not proven whether MT induced by some metals such as bismuth prevented the renal toxicity of *cis*-DDP, because the possibility that metal compounds induce protective factors against *cis*-DDP toxicity other than MT cannot be ruled out. In the present studies, *cis*-DDP nephrotoxicity was prevented by pretreatment with bismuth and zinc compounds in the wild-type control mice, but not in the MT-null mice. In the MT-null mice, the renal MT levels were not increased by either bismuth or zinc treatment, although they were increased in the wild-type control mice by these treatments. These results clearly indicate, therefore, that preinduction of MT synthesis in the target organs protects mice against the renal toxicity of *cis*-DDP.

The mechanisms involved in the protection against *cis*-DDP nephrotoxicity by endogenous MT and its induction are still unclear. However, the binding of platinum to MT has been reported to occur in the liver and kidney of *cis*-DDP-treated rats [26, 29]. Zelazowski et al. [29] have suggested that the binding of the platinum of *cis*-DDP is associated with the removal of zinc from zinc-bound MT. The detoxification of *cis*-DDP by preinduction of renal MT may be explained by the replacement of zinc or bismuth in the renal MT by platinum, which is consequently trapped firmly by the MT. MT can also be involved in protection against oxidative stress and can act as a free radical scavenger [22]. Lazo et al. [12] have recently shown that the sensitivity of MT-null cells to reactive oxygen species is enhanced compared with wild-type cells. Further, *cis*-DDP induces lipid peroxidation in vitro and in vivo, and this is reduced by antioxidants [6, 7]. Boogaard et al. [3] have reported that treatment of rats with *cis*-DDP increases the renal superoxide dismutase activity but this increase does not occur in rats that have received bismuth prior to *cis*-DDP. This suggests that peroxidation contributes to *cis*-DDP-induced nephrotoxicity and that the antioxidant properties of MT are responsible for the reduction of this toxicity. Thus, the sequestration of *cis*-DDP or its metabolites by MT molecules and the antioxidant properties of MT have been proposed as possible mechanisms of detoxification.

In conclusion, we found that induced MT as well as endogenous MT can protect mice against the severe kidney damage caused by *cis*-DDP. The results suggest that MT is an important regulatory factor against the appearance of *cis*-DDP nephrotoxicity which can limit

the clinical use of *cis*-DDP. Patients treated with cancer chemotherapy including *cis*-DDP often show a large variation in the appearance and degree of nephrotoxicity. Further studies are necessary to establish whether differences in endogenous renal MT levels or in genetic traits can explain this variation.

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