Cisplatin-Loaded Polymer-Metal Complex Micelle with Time-Modulated Decaying Property as a Novel Drug Delivery System

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Purpose. The pharmacological activity and pharmacokinetics of cisplatin (CDDP)-loaded polymeric micelles were examined to reveal their usefulness as a novel tumor-directed drug carrier system of CDDP.

Methods. In biodistribution assay, free CDDP or CDDP-loaded micelles were administered intravenously to Lewis lung carcinoma-bearing mice. Antitumor activity and nephrotoxicity were respectively evaluated by the measurement of tumor size and plasma blood urea nitrogen (BUN) after single bolus i.v. administration of each drug.

Results. The time profile of the plasma Pt level after the injection of the micelles exhibited a time-modulated disappearance as observed in saline *in vitro*. The micelles exhibited 5.2- and 4.6-fold higher AUC of Pt in the plasma and tumor, respectively, with minimal change in the kidney, in comparison with free CDDP, suggesting that prolonged circulation of Pt in circulation and specific accumulation in the tumor were achieved utilizing the micellar drug carrier system. Administration of the micelles at the dose exhibiting antitumor activity similar to free CDDP did not increase the plasma BUN, whereas free CDDP induced its remarkable increase.

Conclusion. CDDP-loaded micelles restrained nephrotoxicity, which is the dose-limiting factor of CDDP, while exhibiting tumor-specific accumulation. Thus, CDDP-loaded micelles are expected to be a novel formulation of CDDP for clinical use.

KEY WORDS: cisplatin; drug delivery system; polymeric micelle; biodistribution.

INTRODUCTION

Cisplatin (cis-dichlorodiammineplatinum(II); CDDP) is one of the most widely used antitumor drugs in the treatment of a variety of solid tumors (1). However, its clinical use has a limitation due to its severely toxic side effects, such as acute nephrotoxicity and chronic neurotoxicity (2). Many studies have focused on developing a CDDP analogue that has a reduced toxicity with enhanced antitumor activity in CDDP treatment (3). Nevertheless, new compounds capable of completely avoiding the toxicity of platinous drugs has not been obtained yet.

It is well known that intravenously administered CDDP is rapidly distributed to the whole body and that the greater part goes through glomerular filtration in the kidney (4,5). The interaction of CDDP with serum proteins is considerably slower in comparison with its biodistribution (4). A part of the CDDP filtered through the glomerulus undergoes reabsorption into the body at the tubular duct (4), and such interaction of CDDP with the renal tubules is one of the most likely factors in its nephrotoxicity restricting the therapeutic window of CDDP. Development of a drug delivery system (DDS) that can enhance CDDP accumulation in the tumor while reducing it in the kidney can be a promising approach to achieving enhanced antitumor activity as well as reduced nephrotoxicity in the clinical use of CDDP.

Recently, tumor-directed DDS utilizing prodrugs (6) and microparticulates such as liposomes (7) and micelles (8) has been developed. The development of DDS for CDDP, however, has not been fully completed due to the unfavorable properties of CDDP. For example, the liposomal formulations containing CDDP rapidly leak their contents during storage or within the bloodstream due to the low compatibility between the lipid bilayer and the free drug, although attempts to overcome such a difficulty have been improved (9). To avoid leakage of the free drug from the drug carrier, most current formulations adopt a coordination bond between CDDP and carboxylic group-containing polymers (10, 11) or lipids (12). However, this coordination bond undergoes ligand substitution with the various ions and/or amino acid residues of proteins, resulting in the dissociation of the Pt conjugate. To improve the stability of the conjugation and control the release profile of Pt, several formulations utilizing polymers containing a multicarboxylic group, which can form a stable chelate complex with CDDP (13), β-cyclodextrin, which can form a stable complex with CDDP (14), or an enzymatically degradable bond for the CDDP linkage (15) have been investigated.

We previously reported CDDP-loaded block copolymer micelles that were formed through the complexation between CDDP and poly(ethylene glycol)-poly(aspartic acid) block copolymer (PEG-P(Asp)) in an aqueous medium and characterized its physicochemical properties as a novel drug carrier for CDDP (16,17). The polymer-metal complex in CDDP-loaded micelles could be stable against the ligand substitution reaction by ions and proteins because they form a water-free stable micellar core. Also, the poly(ethylene glycol) (PEG) chain, the corona of the micelle, could prevent serum proteins from interacting with the micellar core. The polymeric micelle drug carrier system is expected to have a long circulation in the bloodstream and eventually to accumulate in the solid tumor. Indeed, the polymeric micelles composed of a corona of PEG and a core of doxorubicin-(Dox)-conjugated poly(aspartic acid) with physically entrapped Dox have shown sufficiently high *in vivo* antitumor activity to completely regress the solid tumor (8,18–20). Furthermore, we have already confirmed that CDDP-loaded micelles reduced such nephrotoxicity and did not show such denuded intestinal epithelium as observed for CDDP in rats, whereas they demonstrated similar antitumor activity to CDDP in human gastric carcinoma-bearing nude mice (personal communication).

In this study, to support the effectiveness of CDDP-

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loaded micelles in terms of the antitumor activity and the toxic side effects, and to demonstrate their usefulness as a tumor-targetable DDS, both the systemic elimination and the biodistribution to the major organs were studied for CDDP-loaded micelles. The present pharmacokinetic study revealed the tumor-specific distribution of CDDP-loaded micelles while avoiding the accumulation and cytotoxic activity of CDDP in the kidney.

MATERIALS AND METHODS

Materials

CDDP was purchased from Aldrich Chemical Co. (Milwaukee, WI). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), blood urea nitrogen (BUN)-UV test kit, GOT-UV, and GPT-UV test-kits were purchased from Wako Pure Chemical Co. (Osaka, Japan). PEG-P(Asp) was synthesized according to the previously reported procedure (17). Briefly, the polymerization of the N-carboxy anhydride of β-benzyl L-aspartate (BLA-NCA) was initiated by the terminal amino group of α-methoxy-ω-aminopoly(ethylene glycol) (CH_3O-PEG-NH_2; $M_w = 5{,}000$). The deprotection of the benzyl group was conducted by mixing in 0.5N NaOH. The polymerization degree of P(Asp) was determined to be 40 by ^{1}H-NMR. Lewis lung carcinoma (LLC) cells were kindly supplied from the National Cancer Center (Japan). Male C57BL/6N mice (20–23 g body weight, 6 weeks old) and female C57BL/6N mice (17–20 g body weight, 6 weeks old) were purchased from Clea Japan (Tokyo, Japan). The former were used for biodistribution and evaluation of toxic side effects, whereas the latter were for the antitumor activity assay. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Methods

Preparation of CDDP-Loaded Polymeric Micelles (17)

PEG-P(Asp) and CDDP were dissolved in distilled water and reacted for 72 h. The CDDP-loaded micelles thus prepared were purified by ultrafiltration (MWCO: 100,000) and then characterized by dynamic light scattering (DLS) measurement to evaluate the size distribution. The Pt content in the micelle was determined by flameless atomic absorption spectrophotometer (AAS) Z-8000 (Hitachi Instruments, Japan).

Cell Culture and Cytotoxicity Assay

In vitro cytotoxicity against LLC cell line, was evaluated by MTT assay. The cell line was maintained as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL Co. [Grand Island, NY]) containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. LLC cells were exposed to the media containing each drug at a different concentration for 24, 48, and 72 h before the MTT assay.

Biodistribution

Mice ($n = 4$) were inoculated subcutaneously on the right flank with LLC cells (1×10^6). CDDP or CDDP-loaded micelles were administered intravenously at a dose of 4.7mg/kg on a CDDP basis 10 days after the inoculation (tumor weight: 0.13±0.04 g [mean±SD]). The animals were sacrificed after defined time periods (1, 8, 15, 30, min; 1, 4, 8, 24, 48, and 72 h for free CDDP and 1, 8, 15, 30 min; 1, 4, 8, 24, 48, and 72 h for the micelles). The tumor, kidney, liver, spleen, and muscle were excised, and blood was collected from the inferior vena cava, heparinized, and centrifuged to obtain the plasma. The plasma and each of the organs were decomposed on heating in nitric acid, evaporated and redissolved in 2 N hydrochloric acid solution to prepare a sample for AAS measurement. The area under the curve (AUC) for plasma and tissue Pt concentration was calculated based on the trapezoidal rule up to 72 h. The tissue-to-plasma concentration ratio (K_p) was calculated by dividing the tissue Pt concentration by its plasma concentration. The distribution volume of the central compartment (V_0) was calculated as the dose divided by the initial plasma concentration, which was extrapolated from the first and second sampling points.

Antitumor Activity

LLC cells (1×10^6) were inoculated into the right flank of mice ($n = 4$) and allowed to grow as a palpable tumor for 3 days. Animals were treated with a single bolus i.v. administration of free CDDP at a dose of 6mg/kg or CDDP-loaded micelles at doses of 8 and 15 mg/kg on CDDP basis. This dose of free CDDP was adopted as a maximum tolerated dose on a single shot because treatment of all animals ($n = 6$) with CDDP at a dose of 11 mg/kg resulted in toxic death. The antitumor activity of each drug was evaluated by measuring the tumor size and body weight change on defined days after drug injection (day 0). Tumor volume was estimated by (21):

$$V = (a) \times (b)^2/2 \quad (1)$$

where a = length and b = width of tumor measured by a caliper. Mice were terminated on the nearest whole day when the tumors became ulcerated.

Evaluation of Nephrotoxicity and Hepatotoxicity

Four days after the mice ($n = 4$) were treated with a single i.v. administration of free CDDP or the micelles, plasma samples were collected. The BUN, glutamic-oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) values in the plasma were determined using test kits (purchased from Wako Pure Chemical Co. [Osaka, Japan]).

RESULTS

Characterization of CDDP-Loaded Polymeric Micelle

CDDP-loaded micelles were prepared through the polymer-metal complexation between CDDP and PEG-P(Asp) in distilled water, and the hydrodynamic diameter (d_h) and polydispersity index (μ_2/Γ^2) were determined as described previously (17). These values were 18.3 nm and 0.133, respectively, by DLS measurement.

In Vitro Cytotoxicity

The *in vitro* cytotoxicity of CDDP or CDDP-loaded micelles against LLC after 24, 48, and 72 h incubation was evaluated as the 50% inhibitory concentration (IC_{50}) shown in Table I. The CDDP-loaded micelles showed an 7~13-fold higher IC_{50} than the free drug at each incubation time (Table I). The IC_{50} of CDDP-loaded micelles preincubated in physiological saline at 37°C for 48 h prior to contact with cells was comparable to that of the free drug (Table I).

Biodistribution

Figure 1 shows the platinum concentration in the plasma (A), tumor (B), kidney (C), and liver (D) after i.v. administration of the drugs. Free CDDP disappeared rapidly from circulation and was distributed to each organ. Especially, rapid and high accumulation was observed in the kidney (Fig. 1C inset). In contrast, after the administration of CDDP-loaded micelles, the platinum level in the plasma was stable up to 4~8 h (61%), followed by a gradual decrease after that time (4~8 h) (Fig. 1A). V_0 was calculated to be 315 and 70.3 ml/kg for free CDDP and CDDP-loaded micelles, respectively. In the tumor, CDDP-loaded micelles exhibited the highest accumulation (sixfold higher than free CDDP) at 8 h (Fig. 1B). The platinum level in the tumor after micelle administration remained approximately threefold higher than that of free CDDP even after 72 h, although the level continuously decreased (Fig. 1B). In the kidney, in which the most serious toxic side effect is observed in CDDP treatment, the CDDP-loaded micelles did not exhibit rapid accumulation of platinum up to 15 min (Fig. 1C inset), followed by accumulation comparable with that of free CDDP (Fig. 1C).

To clarify the kinetics of platinum distribution to each tissue, the time profile of the K_p value was evaluated (Fig. 2). A remarkable difference in this profile was observed between free CDDP and CDDP-loaded micelles (Fig. 2). The micelles exhibited lower K_p (<0.7) in all organs examined up to 8 h followed by a subsequent gradual increase in the liver and spleen, whereas free CDDP showed quite prompt increase in K_p value especially in the kidney and liver within 4 h (Fig. 2). Irrespective of time, CDDP-loaded micelles exhibited a lower K_p for the kidney than free CDDP. However, a higher K_p for the spleen and an almost equivalent K_p for the tumor and liver was observed after 8 h in comparison with free CDDP.

To characterize the exposure of platinum to each tissue, its AUC was evaluated (Table II). The AUC in the plasma and tumor was higher after CDDP-loaded micelle injection, whereas a minimal difference in AUC was found in the kidney (Table II).

Table I. Cytotoxic Activity of CDDP, CDDP-loaded Micelle, and CDDP-Loaded Micelle Preincubated in 0.15 M NaCl Solution for 48 H (Prior to Contact with Lewis Lung Carcinoma Cells

Exposure time (h)	IC_{50} (μg/ml)[a]		
	CDDP	CDDP-loaded micelle	Preincubated micelle
24	7.4	94	17
48	3.8	28	6.1
72	3.4	24	7.2

[a] Fifty percent inhibitory concentration evaluated by MTT assay.

Antitumor Activity

The changes in relative tumor volume and body weight in LLC-bearing mice after single bolus i.v. administration of each drug are shown in Fig. 3A and B, respectively. The CDDP-loaded micelles suppressed tumor growth more effectively than the free CDDP up to 2~4 days (Fig. 3). After 4~6 days, the difference in the tumor growth rate between CDDP and micelle-treated mice gradually disappeared. On day 8, both the free CDDP and CDDP-loaded micelles (at a dose of 8 mg/kg) displayed significant antitumor activity vs. the control ($P<0.005$ and $P<0.002$, respectively), although no statistical difference between free CDDP- and micelle-treated groups was observed (Fig. 3A). The CDDP-loaded micelle treatment at 8 mg/kg showed a minimal decrease in body weight, whereas that at 15mg/kg and free CDDP caused body weight loss up to 6% (Fig. 3B).

Nephrotoxicity and Hepatotoxicity

Table III summarizes the BUN, GOT, and GPT values in mice after i.v. administration of each drug. The free CDDP-treated groups had significant increases in BUN, a nephrotoxic marker, vs. the micelle-treated and untreated groups ($P<0.001$), whereas no statistical difference between the micelle-treated and untreated groups was observed (Table III). The injections of free CDDP or CDDP-loaded micelles both led to a significant increase in GOT vs. no treatment ($P<0.05$) without any change in the GPT (Table III).

DISCUSSION

CDDP is known to exhibit a wide antitumor spectrum but to show rapid distribution to several organs including the kidney and liver after i.v. administration (Fig. 1). CDDP-loaded micelles, a novel drug carrier of CDDP, reduced this accumulation in the kidney (Table II) in spite of its higher plasma AUC value, resulting in minimal nephrotoxicity, which is a dose-limiting factor of CDDP (Table III), while achieving higher accumulation in the tumor (Fig. 1B) and an antitumor effect statistically equivalent to that of CDDP (Fig. 3A). These results demonstrate the specific delivery of platinum to the tumor, compared with the kidney, using this novel carrier system.

We previously reported that the reaction of CDDP and PEG-P(Asp) in an aqueous medium leads to the spontaneous formation of CDDP-loaded micelles having a size of approximately 20 nm with a narrow size distribution (17). In physiological saline, the micelles thus prepared dissociate slowly and constantly release Pt compounds after maintaining their apparent molecular weight for approximately 10 h. The CDDP-loaded micelles showed approximately sevenfold higher IC_{50} in comparison with free CDDP (Table I), suggesting that the micellization of CDDP through the polymer-metal complexation reduced the *in vitro* cytotoxic activity of CDDP. After preincubation in physiological saline, however, the CDDP-loaded micelles nearly recovered the cytotoxic activity (Table I), indicating that Pt compounds released from the micelles through the ligand substitution reaction still preserve antitumor activity almost comparable with that of free

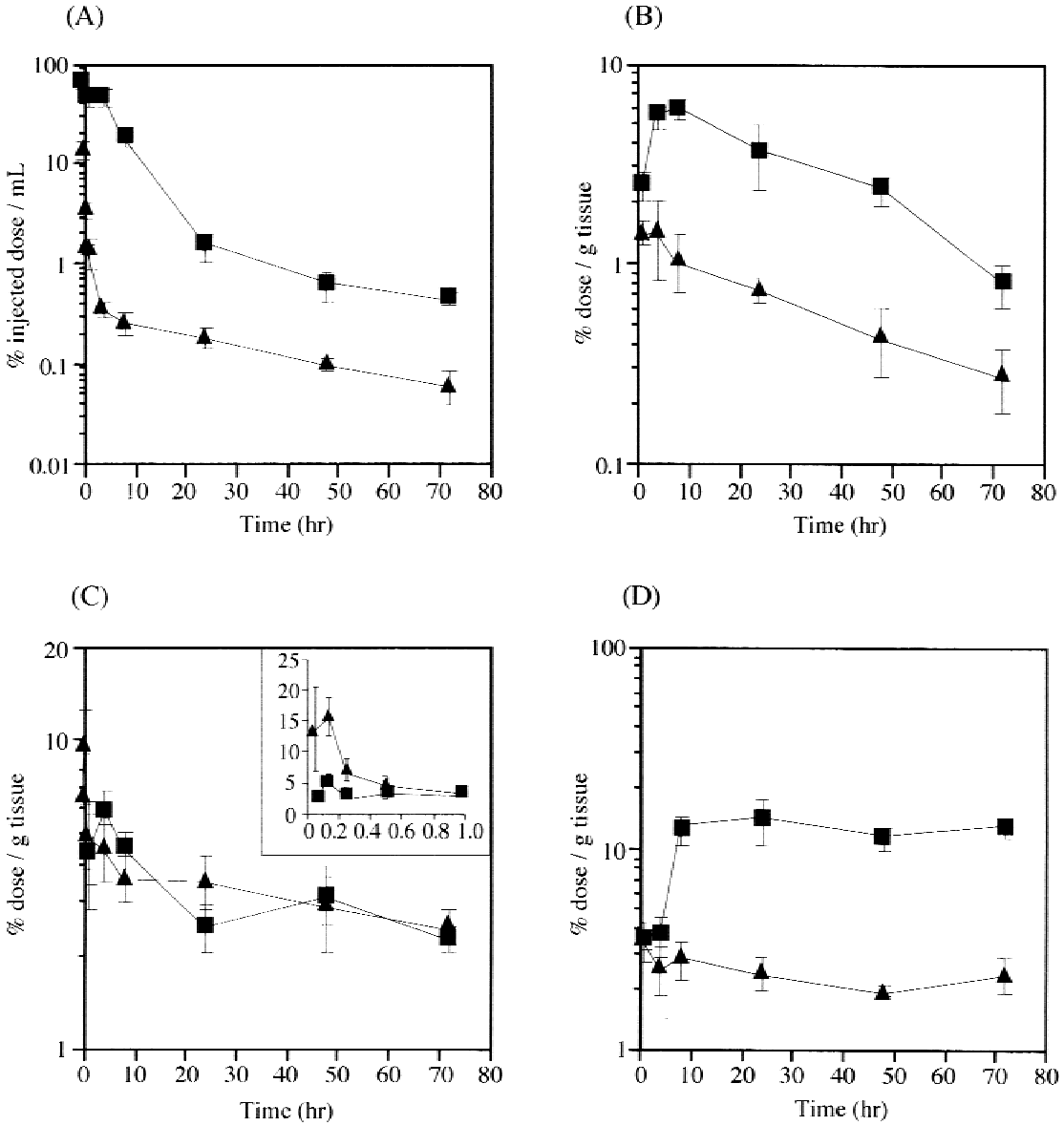

Fig. 1. Time profiles of platinum in plasma (A), tumor (B), kidney (C), and liver (D) in mice ($n=4$) following intravenous bolus injection of CDDP (▲) and CDDP-loaded micelles (■) at a dose of 4.7 mg/kg on CDDP basis. Inset in panel C represents Pt concentration in kidney up to 1 h. Data were normalized by injected dose and presented as mean±SD.

CDDP. Accordingly, CDDP-loaded micelles would be a carrier system that can load CDDP while maintaining the cytotoxic activity.

Biodistribution studies revealed that the platinum in CDDP-loaded micelles in the bloodstream was maintained at a much higher level up to at least 4 h, whereas that of free CDDP disappeared rapidly from circulation (Fig. 1A). The V_0 of CDDP-loaded micelles was not very different from the plasma volume in mice (45.6 ml/kg) (22), indicating that the micelle is stably localized in the plasma compartment after i.v. administration. This result was compatible with the finding that K_p values of CDDP-loaded micelles in the kidney, liver, and spleen are almost equivalent to the vascular space (~0.1ml/g) up to 4 h, suggesting that the micelles are located in the vascular space of the organs (Fig. 2B). This minimal uptake by the tissues is also a characteristic feature of long-circulating liposomes or micelles with a PEG chain on the surface (9,20). In contrast, free CDDP exhibited much higher K_p values especially in the kidney and liver immediately after drug administration, indicating the rapid distribution of CDDP to those organs (Fig. 2A). Such a rapid distribution of platinum, which may be associated with the toxic side effect of CDDP (4), can be reduced by micelle formation (Fig. 2B). An increase in the K_p of CDDP-loaded micelles in all organs was observed after 8 h (Fig. 2B), especially in the liver and spleen, whereas the Pt concentration in plasma (Fig. 1A) started to disappear after 4~8 h. This substantial platinum uptake by the tissues after such a time period can be explained by the beginning of disruption of the micelle structure, because we previously observed *in vitro* that the dissociation of the micelles occurred after a lag period of approximately 10 h in physiological saline at 37°C (17). From these results, CDDP-

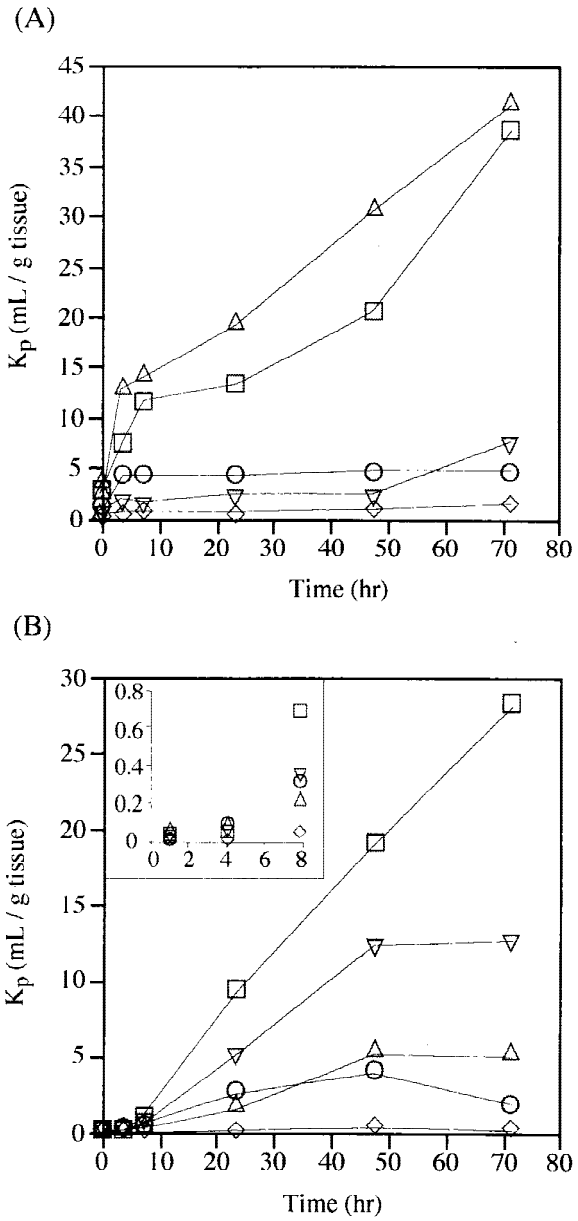

Fig. 2. Time-course of tissue-to-plasma concentration ratio of platinum (K_p) for tumor (○), kidney (△), liver (□), spleen (▽), and muscle (◊) in mice ($n=4$) after injection of CDDP (A) or CDDP-loaded micelles (B) at a dose of 4.7 mg/kg on CDDP basis. Inset in panel B represents the K_p up to 8 h.

loaded micelles are expected to accumulate platinum in the tissues after the disruption of the micelle structure.

The AUC for platinum was calculated from the results of Fig. 1 to characterize its accumulation after the injection of CDDP-loaded micelles (Table II). A 5.2-fold increase in the plasma AUC and a 4.6-fold increase in the tumor AUC were obtained for CDDP-loaded micelles in comparison with the free drug, whereas the kidney AUCs were similar (Table II). Regarding the ratio of the tumor AUC to the kidney AUC, a parameter representing the drug-targeting efficiency for each tissue (21), the micelles showed 4.9-fold higher value than the free drug (Table II). Thus, the accumulation of Pt in the tumor, compared with the kidney, was achieved utilizing the micellar drug carrier system. However, the ratio of the tumor

Table II. Area Under the Plasma and Tissue Concentration Time Curves of Platinum After i.v. Administration of CDDP Alone or CDDP-Loaded Micelle

	CDDP	CDDP-loaded micelle
Plasma[a]	15.7	81.8
Tumor[b]	47.2	220
Kidney[b]	238	225
Liver[b]	167	841
Spleen[b]	21.4	460
Muscle[b]	7.00	11.1

[a] Unit: μg*h/ml.
[b] Unit: μg*h/g tissue.

AUC to the plasma AUC was not so much different between free CDDP and CDDP-loaded micelles (Table II), suggesting that the effective accumulation of the micelles in tumor is mainly due to their prolonged transit time in circulating plasma. On the other hand, regarding the ratio of the kidney AUC to the plasma AUC, the micelles had less than one-fifth value of the free drug (Table II). This lower value of the micelles might be ascribed to reduced glomerular filtration of micellar CDDP, whereas free CDDP easily undergoes glomerular filtration and subsequently interacts with the tubular duct, exhibiting higher Pt concentration (Fig. 1C inset) and K_p (Fig. 2A) in the kidney just after the injection. It is reported that the BUN level is related to the AUC calculated by the plasma concentration of unchanged CDDP being greater than the threshold plasma concentration level, regardless of the CDDP dose and schedule (23). CDDP-loaded micelles did not show such rapid and high accumulation of Pt in the kidney as observed for CDDP within 15 min, which may be one of the reasons for the restrained increase in BUN level for the micelle-treated animals as summarized in Table III. It should be noted that CDDP-loaded micelles did not cause an increment in the K_p value for the kidney even after the micelle structure was assumed to be disrupted (4~8 h) (Fig. 2).

CDDP-loaded micelles had an antitumor activity at least comparable to that of free CDDP in this limited dose regimen (single bolus injection) (Fig. 3A). This antitumor activity, however, seems to be in conflict with the high accumulation of micelles in the tumor, because the micelles showed a 4.6-fold higher AUC than free CDDP (Table II). This may be explained if we consider that such an AUC of platinum in the tumor at least partially represents the micelles located in the extracellular space. The K_p value in the tumor up to 8 h, when the tumor platinum concentration reached a maximum, was at most 0.35 ml/g tumor (Fig. 2B). On the other hand, the vascular volume and the interstitial volume in tumor is reported to be 0.07 and 0.55ml/g tumor, respectively (24). Accordingly, a large part of the platinum accumulated in the tumor up to 8 h is considered to be located in the vascular and/or or the interstitial space, possibly due to the enhanced permeability and retention (EPR) effect (25,26). Considering that CDDP-loaded micelles had reduced *in vitro* cytotoxicity in comparison with the free drug (Table I), it may be reasonable that the AUC found in the tumor does not quantitatively account for the antitumor activity. Considering that the micelles are stably accumulated in the extracellular space of the tumor during the lag time before the micelles are dissociated, the development of micelles having more stable characteris-

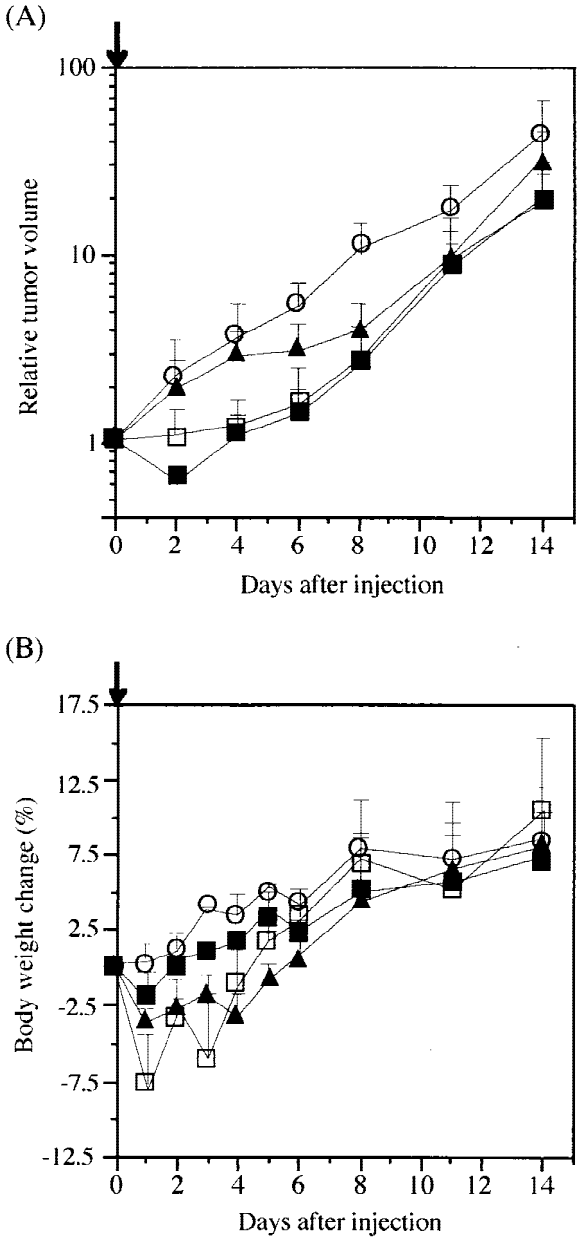

Fig. 3. Relative tumor size in abdominal skin (A) and changes in body weight (B) in C57BL/6N mice ($n=4$). Treatments were performed by single i.v. administration on 0 day (arrowhead). Data are presented as mean±SD. (○: control; ▲: CDDP 6mg/kg; ■: CDDP-loaded micelles 8mg/kg; □: CDDP-loaded micelles 15mg/kg, the dose of the micelles is represented by the CDDP basis.)

tics in the bloodstream might allow the drug to be accumulated in the tumor at a much higher level. The structural decay in the bloodstream of the micelles prepared in the present study resulted in a higher platinum accumulation in the liver (Fig. 1D and 2B), which may be associated with the comparable increase in GOT with free CDDP (Table III). Therefore, the improvement in micelle stability with the prolonged incubation period necessary for the decay under physiological conditions is expected to lower the undesirable accumulation in the normal tissues as well as enhance accumulation in tumor, leading to the development of a useful drug carrier system for clinical CDDP treatment.

Table III. Values of Nephrotoxic and Hepatotoxic Markers in Normal and Drug-Injected Mice

	BUN[a]	GOT[a]	GPT[a]
Normal[b]	25.5 ± 1.65	67.5 ± 3.1	37.8 ± 6.65
CDDP[c]	36.5 ± 2.71[e]	94.8 ± 11.1[e]	41.8 ± 4.27
Micelle[d]	25.5 ± 1.22[f]	90.0 ± 7.02[e]	38.8 ± 4.65

Note. Each marker was measured 4 days after administration of CDDP or CDDP-loaded micelle (mean ± SD, $N = 4$).
[a] Unit: mg/dl.
[b] Untreated mice.
[c] 6 mg/kg.
[d] 8 mg/kg on CDDP basis.
[e] Significantly different from normal.
[f] Significantly different from CDDP.

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