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Novel Cisplatin-Incorporated Polymeric Micelles Can Eradicate Solid Tumors in Mice

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ABSTRACT

Polymeric micelles incorporating cisplatin (CDDP) were prepared through the polymer-metal complex formation between CDDP and poly(ethylene glycol)-poly(glutamic acid) block copolymers, and their utility as a tumor-targeted drug delivery system was investigated. CDDP-incorporated micelles (CDDP/m) had a size of 28 nm with remarkably narrow distribution. CDDP/m were very stable in distilled water even in long-time storage, but exhibited a sustained drug release accompanied with the decay of the carrier itself in physiological saline. These micelles showed remarkably prolonged blood circulation and effectively accumulated in solid tumors (Lewis lung carcinoma cells) according to the passive targeting manner (20-fold higher than free CDDP). Reduced accumulation of the micelles in normal organs provided high selectivity to the tumor. *In vivo* antitumor activity assay demonstrated that both free CDDP and the CDDP/m had significant antitumor activity in C 26-bearing mice compared with nontreatment ($P < 0.05$ for free CDDP; $P < 0.01$ for CDDP/m), but complete tumor regression was observed only for the treatment with CDDP/m. Four of 10 mice treated with CDDP/m (4 mg/kg; five times administration at 2-day intervals) showed complete tumor regression with no significant body weight loss, whereas free CDDP treatment at the same drug dose and regime resulted in tumor survivals and ~20% of body weight loss. These data suggest that CDDP/m could be a promising formulation of CDDP for the targeted therapy of solid tumors.

INTRODUCTION

Cisplatin [*cis*-dichlorodiammineplatinum(II); CDDP] is an important class of antitumor agents and is widely used for the treatment of many malignancies, including testicular, ovarian, bladder, head and neck, small-cell, and non-small-cell lung cancers (1). However, the clinical use of this drug is limited due to emergence of intrinsic and acquired resistance (2), and severe side effects, such as acute nephrotoxicity and chronic neurotoxicity (3). There might be two potential methods for improving the therapeutic indices of this drug, *i.e.*, the screening of more potent platinum analogues such as carboplatin and oxaliplatin (4) and the development of tumor-targetable formulations of platinous drugs (5), including the use of macromolecular carriers, such as water-soluble polymers (6–9), long-circulating liposomes (10–12), and polymeric micelles (13–15). It is well accepted that macromolecular carriers potentially exhibit prolonged blood circulation with reduced nonspecific accumulation in normal tissues and preferential tumor accumulation due to the enhanced permeability and retention (EPR) effect, characterized by microvascular hyperpermeability to circulating macromolecules and impaired lymphatic drainage in tumor tissues (16–18). Indeed, some macromolecular carrier systems incorporating antitumor agents have been approved for clinical use (19) or are being studied currently in clinical trials (20, 21). Despite the aforementioned advantages of macromolecular carriers, improved therapeutic efficacy, which can cause complete tumor regression, has been demonstrated for limited species of drugs such as doxorubicin (22–27), probably due to difficulties in preparing the optimal formulations. For instance, for CDDP and its derivatives, their unfavorable properties such as poor water solubility and low lipophilicity may hamper the development of their optimal formulations. Hence, additional efforts are needed to be devoted to the development of the useful macromolecular carrier systems incorporating platinous antitumor drugs.

Polymeric micelles have received considerable attention recently as a promising modality of macromolecular carrier systems (26, 28, 29). It has been demonstrated that polymeric micelles could circulate stably in the bloodstream and, therefore, accumulate effectively in the solid tumor due to the aforementioned EPR effect (15, 26). The doxorubicin-loaded polymeric micelles have shown remarkably high antitumor activity causing complete tumor regression (26), and have progressed recently to Phase I clinical trial at the National Cancer Center Hospital (Tokyo, Japan). Furthermore, Torchilin *et al.* (30) has demonstrated recently that antibody-conjugated polymeric micelles (immunomicelles) showed the improved tumor accumulation of poorly soluble drugs, resulting in better therapeutic effects. Thus, polymeric micelle-based drug carriers might possess several unique advantages (28, 29), including: (*a*) the applicability to use a variety of therapeutic agents such as hydrophobic compounds, metal complexes, and charged macromolecules [*e.g.*, proteins (31) and DNA (32–34)]; (*b*) unnecessariness of chemical structure modification of the drugs; (*c*) simplicity of micelle preparation; (*d*) high drug loading capacity; (*e*) controlled drug release; (*f*) small size to allow deeper penetration into poorly permeable tumors (35, 36); and (*g*) possible modulation of the micellar surface with active-targeting ligands (30, 37–40). These properties could be optimized by modulating the micelle-forming block copolymers depending on the chemical and physicochemical properties of the drugs.

We recently developed a new class of polymeric micelles incorporating CDDP via the polymer-metal complex formation between CDDP and poly(ethylene glycol)-poly(aspartic acid) block copolymers [PEG-P(Asp)] in an aqueous medium as a potential tumor-selective carrier for CDDP (13–15). A coordination bond between the platinum(II) atom and a carboxylic group in the side chain of P(Asp) could allow regeneration of free drug in the presence of abundant chloride ions. Indeed, CDDP-incorporated micelles showed slow dissociation into unimers, accompanied with a sustained CDDP release in physiological saline, whereas the micelles were extremely stable in distilled water (14). When administered i.v. to tumor-bearing mice, the micelle showed 5.2- and 4.6-fold higher area under the platinum (Pt) concentration-time curve for the plasma and tumor, respectively, in comparison with free CDDP (15). However, the fast structural decay of the micelles caused the liver and spleen accumulation at a

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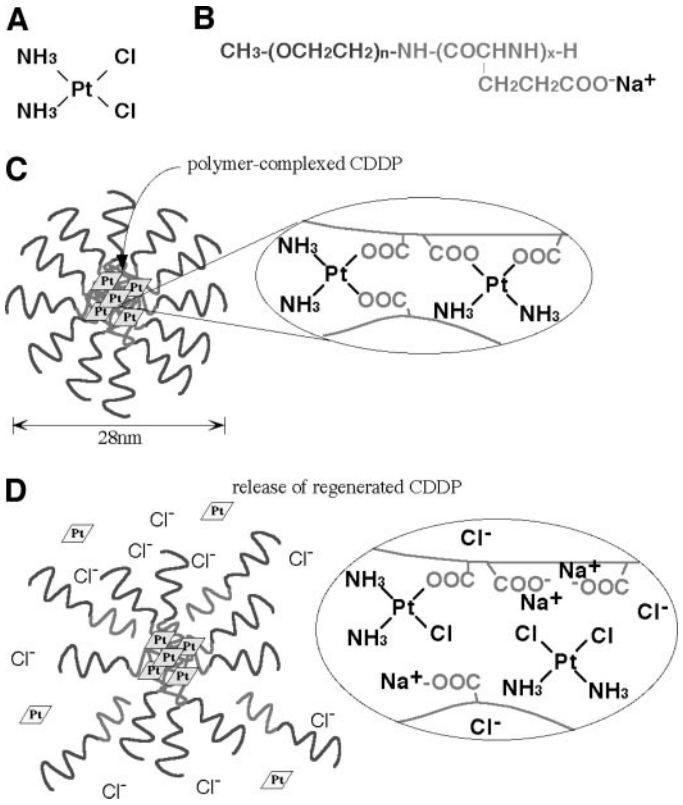

Fig. 1. Chemical structures of CDDP (*A*) and PEG-P(Glu) block copolymers (*B*), and schematic illustrations of CDDP-incorporated micelles (*C*) and the hypothesized behavior of the micelles in physiological saline at 37°C (*D*). The CDDP-incorporated micelles are spontaneously formed via a ligand exchange reaction of Pt(II) from the chloride to the carboxylates in the copolymers in distilled water (*C*), and the micelles dissociate accompanied with the sustained release of CDDP via an inverse ligand exchange reaction of Pt(II) from the carboxylates in the copolymer to the chloride ions in the surroundings in physiological saline (*D*; Ref. 14).

higher level, resulting in comparable antitumor activity to free CDDP despite restrained nephrotoxicity (15). In this study, CDDP-incorporated micelles were newly prepared through the complexation between CDDP and poly(ethylene glycol)-poly(glutamic acid) block copolymers [PEG-P(Glu)] to improve and optimize the micellar stability, as well as the drug release profile, which are likely determining factors of the drug disposition in the body (Fig. 1). This study investigated the biodistribution and *in vivo* antitumor activity of CDDP-incorporated micelles with the optimized properties. We demonstrated that CDDP-incorporated micelles accumulated significantly and selectively in solid tumors, and eventually showed remarkably high *in vivo* antitumor activity causing eradication of the tumors. The CDDP-incorporated micelles might offer a new formulation of platinous drugs with improved therapeutic indices, which is applicable to clinical use.

MATERIALS AND METHODS

Materials. β-Benzyl L-glutamate and bis(trichloromethyl)carbonate (triphosgene) were purchased from Sigma Chemical Co., Inc. (St. Louis, MO) and Tokyo Kasei Kogyo Co., Inc. (Tokyo, Japan), respectively. *N*,*N*-Dimethylformamide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Wako Pure Chemical Co., Inc. (Osaka, Japan). CDDP was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). α-Methoxy-ω-aminopoly(ethylene glycol; CH_3O-PEG-NH_2; M_w = 12,000) was a kind gift from Nippon Oil and Fats Co., Inc. (Tokyo, Japan).

Cell Lines and Animals. Lewis lung carcinoma (LLC) and murine colon adenocarcinoma 26 (C 26) cells were kindly supplied from the National Cancer Center. LLC cells were maintained in DMEM (Sigma Chemical Co., Inc.) containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. C 26 cells were maintained *in vivo* using BALB/c mice before transplantation into CDF1 mice. C57BL/6N mice (male; 20–23 g body weight; 6 weeks old) and CDF1 mice (female; 16–20 g body weight; 6 weeks old) were purchased from Clea Japan (Tokyo, Japan) and Charles River Japan (Kanagawa, Japan), respectively. All of the animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH.

Preparation of PEG-P(Glu). PEG-P(Glu) block copolymers were synthesized by the previously reported synthetic method of PEG-P(Asp) with a slight modification (14). Briefly, the *N*-carboxy anhydride of β-benzyl L-glutamate was synthesized by the Fuchs-Farthing method using triphosgene (41). Then, *N*-carboxy anhydride of β-benzyl L-glutamate was polymerized in *N*,*N*-dimethylformamide, initiated by the NH_2 amino group of CH_3O-PEG-NH_2, to obtain PEG-poly(g-benzyl L-glutamate) block copolymers. A narrow distribution (M_w/M_n: 1.06) of PEG-poly(g-benzyl L-glutamate) was confirmed by gel permeation chromatography [column: TSK-gel G3000H_{HR}, G4000H_{HR} (Tosoh Co., Inc., Yamaguchi, Japan); eluent: *N*,*N*-dimethylformamide containing 10 mM LiCl; flow rate: 0.8 ml/min; detector: Refractive Index (RI); temperature: 40°C]. The polymerization degree of PBLG was determined to be 38 by comparing the proton ratios of PEG (-OCH_2CH_2-: δ = 3.7 ppm) and phenyl groups of PBLA (-$CH_2C_6H_5$: δ = 7.3 ppm) in ^{1}H NMR measurement [JEOL EX270 (JEOL Inc. (Tokyo, Japan); solvent: DMSO-d_6; temperature: 80°C]. The deprotection of the benzyl group was performed by mixing with 0.1 N NaOH at ambient temperature to obtain PEG-P(Glu).

Preparation of CDDP-Incorporated Micelles. PEG-P(Glu) and CDDP were dissolved in distilled water [(Glu) = 5 mmol/liter; (CDDP)/(Glu) = 1.0] and reacted for 72 h. CDDP-incorporated micelles thus prepared were purified by ultrafiltration (molecular weight cut-off size: 100,000). The size distribution of CDDP-incorporated micelles was evaluated by dynamic light scattering (DLS) measurement at 25°C using a Photal dynamic laser scattering spectrometer DLS-7000 (Otsuka Electronics Co., Inc., Osaka, Japan). The Pt content in the micelles was determined by flameless atomic absorption spectrophotometer Z-8000 (Hitachi Instruments Co., Inc., Tokyo, Japan).

Drug Release and Micellar Stability in Physiological Saline. The release of the platinum from the micelles in physiological saline [10 mM phosphate buffer (pH7.4) + 150 mM NaCl] at 37°C was evaluated by the dialysis method (Spectra/Por-6; MWCO: 1000), Spectrum Laboratories, Inc. (Rancho Dominguez, CA) as reported previously (14). The released Pt outside of the dialysis bag was sampled at a defined time period, and measured by atomic absorption spectrophotometer.

The stability of CDDP-incorporated micelles in physiological saline at 37°C was evaluated by static light scattering measurement using a DLS-7000 according to the method reported previously (14). Change in the scattering light intensity (*i.e.*, the Rayleigh ratio at 90° of the scattering angle) was measured at a defined time period. In this analysis, a decrease in the scattering light intensity should be due to a decrease in the apparent molecular weight of the micelles as well as in the micelle concentration.

Biodistribution. C57BL/6N mice (n = 4) were inoculated s.c. on the right flank with LLC cells (1×10^6). After 14 days, CDDP and CDDP-incorporated micelles were administered i.v. at a dose of 100 μg/mouse on a CDDP basis (tumor weight: 0.17 ± 0.05 g; mean ± SD). The mice were sacrificed after defined time periods (3 min for the plasma and kidney, and 1, 4, 8, and 24 h for all of the organs). The tumor, kidney, liver, spleen, and muscle were excised, and the blood was collected from the inferior vena cava, heparinized, and centrifuged to obtain the plasma. The plasma and the organs were decomposed on heating in nitric acid, evaporated to dryness, and redissolved in 2 N hydrochloric acid solution. The Pt concentration in the solution was measured by atomic absorption spectrophotometer measurement.

***In Vitro* Cytotoxicity.** The growth-inhibitory activity of free CDDP and CDDP-incorporated micelles on the LLC and C 26 cell lines was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. LLC and C 26 cells (5000 cells) were cultured in DMEM containing 10% fetal bovine serum in a 96-well multiplate. The cells were then exposed to each drug (free CDDP or CDDP-incorporated micelles) for 48 or 72 h, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was added. Cell viability was measured by the formed formazan absorbance at 570 nm.

***In Vivo* Antitumor Activity.** A small fragment of C 26 tumors, which were maintained *in vivo* using BALB/c mice, was transplanted s.c. into the right

back of female CDF1 mice. Seven days later (tumor volume: 17.6 ± 1.2 mm^3; mean ± SD), the mice were treated i.v. five times at 2-day intervals with free CDDP at doses of 2 and 4 mg/kg, or CDDP-incorporated micelles at doses of 1, 2, and 4 mg/kg on a CDDP basis. The antitumor activity was evaluated in terms of the tumor size ($n = 10$), which was estimated by the following equation: $V = (a)x(b)^2/2$, where (a) and (b) are major and minor axes of the tumor measured by a caliper, respectively. The body weight was measured simultaneously as an indicator of systemic toxicity. The surviving mice were sacrificed 31 days after the tumor transplantation, and the blood urea nitrogen, a marker of nephrotoxicity, was measured by using Clinical Analyzer 7070 (Hitachi Instruments Co., Inc.). The statistical analysis of animal data were carried out by the Mann-Whitney *U* test.

RESULTS

Characterization of CDDP-Incorporated Micelles. The reaction of CDDP and PEG-P(Glu) in distilled water led to the spontaneous formation of CDDP-incorporated micelles. The Γ-averaged size distribution of CDDP-incorporated micelles measured by the DLS measurement is shown in Fig. 2. It was demonstrated that CDDP-incorporated micelles had a hydrodynamic diameter (d_h) of 28 nm with a remarkably narrow distribution [the polydispersity index (μ_2/Γ^2) by the cumulant method: 0.070]. The drug content in the micelles was determined to be 39% (w/w) by the atomic absorption spectrophotometer measurement, indicating that a remarkably high drug loading capacity was achieved by the micellar formulation. Also, the molar ratios of CDDP to block copolymers and carboxylate groups in the copolymers were estimated to be 27 and 0.71, respectively.

The CDDP-incorporated micelles are extremely stable in distilled water, with no dissociation and precipitation for a prolonged period of time even under very diluted condition (Fig. 1*C*). As described in the introduction, however, CDDP-incorporated micelles could dissociate into unimers, accompanied with CDDP release in chloride ion-rich solutions due to an inverse ligand substitution of the Pt(II) atom from a Glu residue of PEG-P(Glu) to chloride (Ref. 14; Fig. 1*D*). The time courses of the release of Pt from the micelles and the relative scattering light intensity of the micelles in physiological saline at 37°C are shown in Fig. 3, *A* and *B*, respectively. The CDDP-incorporated micelles released the platinum in a sustained manner >150 h, and no initial burst of the drug release was observed. The scattering light intensity concomitantly decreased to ~5% within 100 h. Despite a continued decrease in the scattering light intensity, the hydrodynamic diameter of CDDP-incorporated micelles was kept at >25 nm up to 50 h (Fig. 3*C*). Therefore, the CDDP-incorporated micelles might dissociate gradually while maintaining the hydrodynamic diameter, synchronized with the release of CDDP in physiological saline at 37°C. The time scale of decaying of the micelles was evidently prolonged when compared with the decaying period reported previously (~30 h) of the micelles, which were formed from PEG-P(Asp) (14). The prolonged decaying period of CDDP-incorporated micelles improved the selectivity and efficiency in tumor targeting as described later.

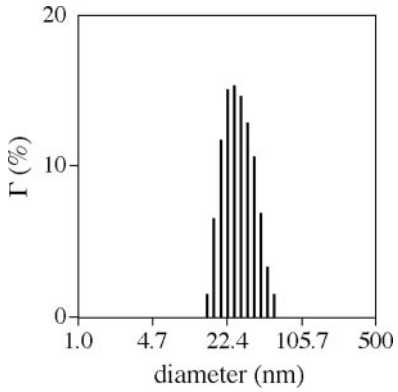

Fig. 2. γ-averaged size distribution of CDDP-incorporated micelles measured by DLS (temperature, 24.5°C; detection angle, 90°).

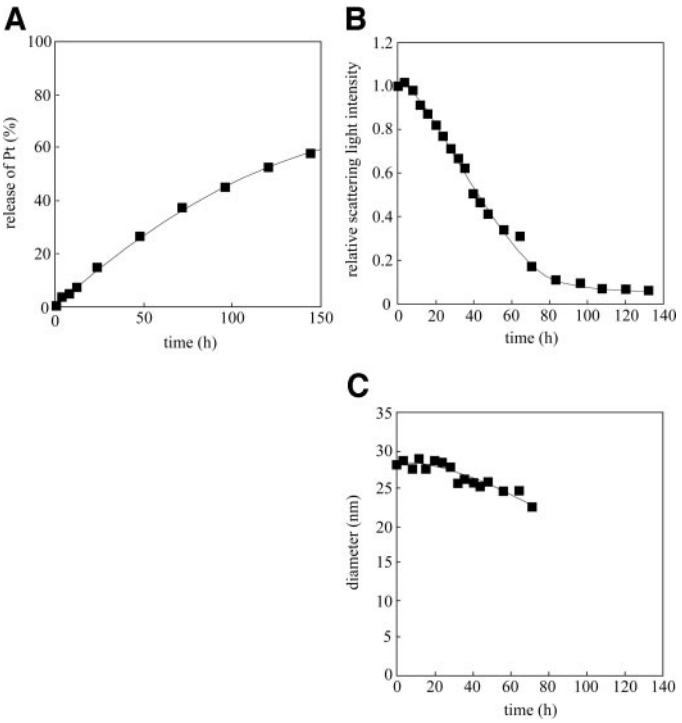

Fig. 3. Time-dependent change in the stability of CDDP-incorporated micelles in physiological saline at 37°C. *A*, release of CDDP from the micelles evaluated by the dialysis method. *B*, change in the relative scattering light intensity of the micelle solution measured at 90° in static light scattering measurement (micelle concentration: 1.5 mg/ml). *C*, change in the hydrodynamic diameter of the micelle solution measured at 90° in DLS measurement. The measurement after 72 h was not performed due to decreased photon counts.

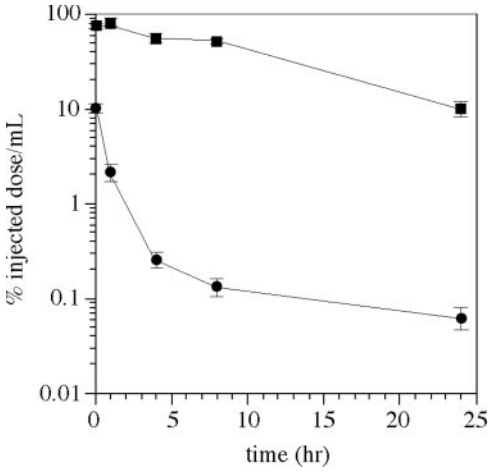

Fig. 4. Time profiles of platinum concentration in the plasma after i.v. administration of free CDDP (●) and CDDP-incorporated micelles (■). Each drug was administered to LLC-bearing mice (male, $n = 4$) at a dose of 0.1 mg on CDDP basis. The molar ratio of CDDP to block copolymer in the micelles was 27, and the dose on the injected polymer was 0.16 mg per a mouse. Data were expressed as mean; *bars*, ±SE.

Plasma Clearance. The plasma Pt level after i.v. administration of free CDDP or CDDP-incorporated micelles is shown in Fig. 4. The CDDP-incorporated micelles showed remarkably prolonged blood circulation, whereas free CDDP was cleared rapidly from circulation.

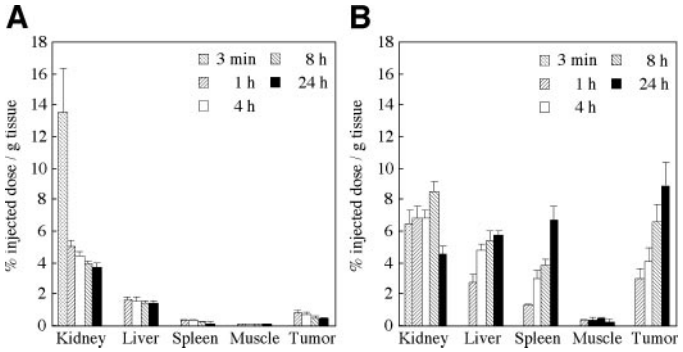

Fig. 5. Tissue distribution of platinum after i.v. administration of free CDDP (*A*) and CDDP-incorporated micelles (*B*). Each drug was administered to LLC-bearing mice (male, $n = 4$) at a dose of 0.1 mg on CDDP basis. The molar ratio of CDDP to block copolymer in the micelles was 27, and the dose on the injected polymer was 0.16 mg per a mouse. Data are expressed as mean; *bars*, ±SE.

Considering the plasma volume in mice (45.6 ml/kg; Ref. 42), CDDP-incorporated micelles maintained >60% of injected dose in the plasma up to 8 h, and ~13% of the plasma Pt level even at 24 h after injection. This plasma Pt level of the micelles was significantly prolonged when compared with the micelles reported previously formed from PEG-P(Asp), which exhibited 1.5% of the injected dose at 24 h after injection (15). It appears that the longevity of CDDP-incorporated micelles in the bloodstream is reasonably correlated with the sustained decaying property of the micelles in physiological saline shown in Fig. 3.

Tissue Distribution. High and selective accumulation of drug carriers at the tumor site is essential for drug targeting success. Accumulation of free CDDP and CDDP-incorporated micelles in normal tissues (kidney, liver, spleen, and muscle) and solid tumor (LLC cells) is shown in Fig. 5, *A* and *B*, respectively. Free CDDP was rapidly distributed to each organ in agreement with its rapid plasma clearance, and the Pt level in each organ reached the maximum within 1 h after i.v. administration. The greater part of free CDDP appeared to be excreted through the glomerular filtration as the highest Pt level was detected in the kidney at 3 min after injection as reported by others (43). It is reported that peak urinary CDDP concentration correlates with nephrotoxicity much better than total renal platinum concentration (44). Therefore, such a rapid accumulation of free CDDP in the kidney may be associated with its renal toxicity. In contrast, the CDDP-incorporated micelles showed cumulative accumulation in each organ and solid tumor due to its remarkably prolonged blood circulation time, and the Pt level in the liver, spleen, and tumor continued to increase up to at least 24 h after injection (Fig. 5*B*). Consequently, the CDDP-incorporated micelle exhibited 4.0-, 39-, and 20-fold higher accumulation in the liver, spleen, and tumor, respectively, than free CDDP at 24 h after injection ($P < 0.01$). On the other hand, the CDDP-incorporated micelles did not exhibit such a high maximum concentration in the kidney as observed for free CDDP at 3 min after injection, suggesting the restrained renal toxicity of the micelles compared with free CDDP.

To assess the selectivity to the solid tumor, the accumulation ratios and area under the Pt concentration-time curve ratios of the tumor to normal tissues at 24 h after injection are summarized in Table 1. The area under the Pt concentration-time curve was calculated based on the trapezoidal rule up to 24 h. In Table 1, free CDDP exhibited the ratios of the tumor to the kidney and liver <0.15 and 0.4, respectively, suggesting its high selectivity to the kidney and liver. In contrast, the CDDP-incorporated micelles exhibited the accumulation and area under the Pt concentration-time curve ratios >1.0, suggesting its selective accumulation in the tumor.

***In Vitro* Cytotoxicity.** Fifty percent cell growth IC_{50} values of free CDDP and CDDP-incorporated micelles against LLC and C 26 cell lines after 48 and 72 h drug incubation are shown in Table 2. The CDDP-incorporated micelles showed lower cytotoxicity than free CDDP at each incubation time. Assuming that the Pt complexes released from the micelle play an essential role in the cytotoxic activity, this range of reduction in cytotoxicity may be consistent with the sustained manner of Pt release shown in Fig. 3*A*. Actually, we reported previously that the dissociation of CDDP-incorporated micelles after preincubation in physiological saline resulted in *in vitro* cytotoxicity nearly comparable with that of free CDDP (15).

***In Vivo* Antitumor Activity.** Although both free CDDP and CDDP-incorporated micelles showed *in vivo* antitumor activity in LLC-bearing C57BL/6N mice (data not shown), the most significant *in vivo* antitumor activity was presently observed when a model of C 26-bearing CDF1 mice was used. In the preliminary study, C 26-bearing CDF1 mice were treated i.v. three times at 2-day intervals with free CDDP at doses of 2.5 and 5 mg/kg or CDDP-incorporated micelles at the same doses on a CDDP basis. The mice treated with 5 mg/kg of free CDDP, and those treated with 2.5 and 5 mg/kg of the micelles showed moderate response to the tumor; however, no mice showed the tumor regression. Hence, in this study, CDF1 mice ($n = 10$) were treated i.v. five times at 2-day intervals with free CDDP at doses of 2 and 4 mg/kg or CDDP-incorporated micelles at doses of 1, 2, and 4 mg/kg on a CDDP basis. The mice treated with 2 mg/kg of free CDDP, and those treated with 1 and 2 mg/kg of the micelles did not show significant tumor growth inhibition ($P > 0.05$), although the treatment with 2 mg/kg of the micelles caused the tumor regression in 2 of 10 mice. The significant tumor growth inhibition was observed for the mice treated with 4 mg/kg of free CDDP ($P < 0.05$ on day 28) and 4 mg/kg of the micelles ($P < 0.01$ on day 28; Fig. 6). Fig. 6 shows the time-dependent change in tumor volume after s.c. transplantation. Each drug was i.v. administered on days 7, 9, 11, 13, and 15. In Fig. 6, 6 of 10 mice treated with CDDP-incorporated micelles showed complete tumor regression after the fifth administration. In those 6 mice showing tumor regression, however, 1 mouse died after day 17, and another 1 showed tumor recurrence after day 28. Consequently, 4 tumors were completely cured in the mice treated with CDDP-incorporated micelles. The complete disappearance of primary tumors in these mice was confirmed by dissection on day 31.

Table 1 *Accumulation ratios and area under the concentration time curve (AUC) ratios between the tumor (Lewis lung carcinoma) and normal organs at 24 h after administration of free cisplatin (CDDP) and the CDDP-incorporated micelles (CDDP/m)*[a]

	Accumulation ratio[b]		AUC ratio[c]	
	CDDP	CDDP/m	CDDP	CDDP/m
Tumor/kidney	0.13 ± 0.02	2.0 ± 0.4	0.13	0.97
Tumor/liver	0.34 ± 0.07	1.6 ± 0.3	0.38	1.3
Tumor/spleen	4.0 ± 1.5	1.3 ± 0.1	2.4	1.5

[a] Dose: 0.1 mg per mouse on CDDP basis.
[b] Mean ± SE ($n = 4$).
[c] AUC was calculated based on the trapezoidal rule up to 24 h.

Table 2 *In vitro cytotoxicity of free cisplatin (CDDP) and CDDP-incorporated micelle (CDDP/m) against LLC and C 26 cell lines*

Cell line	Incubation time (h)	IC_{50} (μg/ml)[a]	
		CDDP	CDDP/m
LLC	48	2.4	26
	72	1.3	16
C 26	48	2.4	20
	72	0.56	7.5

[a] IC_{50} evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

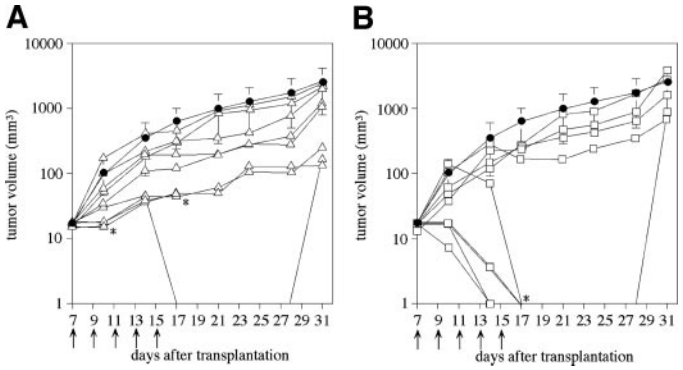

Fig. 6. Effect of free CDDP (△ in *A*) and CDDP-incorporated micelles (□ in *B*) on the growth of C 26 colon adenocarcinoma s.c. transplanted to CDF1 mice (female, $n = 10$). Each drug was administered five times at 2-day intervals (*arrows*) at the dose of 4 mg/kg on a CDDP. The molar ratio of CDDP to block copolymer in the micelles was 27, and the dose on the injected polymer was 6.3 mg/kg. The tumor volume in control mice ($n = 10$) administered with saline administration (●) is expressed as mean; *bars*, ±SE, and that in the drug-treated mice ($n = 10$; △ and □) is presented as the data for individual mice. Significant differences in the tumor volume were found between control and the mice treated with free CDDP ($P < 0.05$), and between control and the mice treated with CDDP-incorporated micelles ($P < 0.01$) on day 28. ∗ in the figures represents death of mice.

In contrast, 1 of 10 mice treated with free CDDP showed tumor regression after day 14, but this mouse showed tumor regrowth after day 28. Two mice died after days 11 and 17. Consequently, no tumor cure was found in the mice treated with free CDDP.

The time course of body weight change, where the body weight on day 7 is normalized to 100%, was simultaneously monitored, and is shown in Fig. 7. The mice treated with free CDDP showed ~20% of body weight loss on day 17 (after the fifth administration), which is significantly less than nontreatment group ($P < 0.005$). In contrast, the mice treated with CDDP-incorporated micelles showed no significant body weight loss when compared with the nontreatment group on the same day ($P > 0.05$). Also, both mice treated with free CDDP and CDDP-incorporated micelles did not show an increase in the blood urea nitrogen value over the normal range (~ 40 mg/dl; Ref. 45) on day 31. It appears that CDDP-incorporated micelles are less toxic than free CDDP despite its remarkably improved therapeutic efficacy as shown in Fig. 6.

DISCUSSION

The CDDP-incorporated micelles are a novel type of micellar formulation for metal complexed drugs with a time-modulated decaying property. This unique property was demonstrated *in vitro* and *in vivo* as shown in Figs. 3 and 4, respectively. The long-circulating property of the micelles was not compromised by its high drug-loading amount [39% (w/w)]. The biodistribution of the micelles may be mainly determined by its surface properties and size, and may be less affected by the properties of the loaded drugs if the drug-loaded core is sufficiently embedded in the PEG layer. The CDDP-incorporated micelle reported here might be well balanced between sufficient stability to prolong circulation in bloodstream and adequate drug release to elicit the pharmacological activity at the delivery site. Accompanied by the CDDP release, the micellar carrier itself dissociated into unimers, allowing excretion of the constituent polymers from the body. We demonstrated previously that the micelle-forming block copolymers could be excreted into urine because of their molecular weight being lower than a threshold value for glomerular excretion (M_r 42,000–50,000 for water-soluble synthetic polymers; Refs. 46, 47). Therefore, the CDDP-incorporated micelles could be a safe vehicle with the least risk of nonspecific accumulation of polymers in the body.

Currently, tumor-targeting therapy is studied by two potential strategies: passive targeting and active targeting. In the passive targeting, the drug carriers are designed to exhibit prolonged blood circulation with reduced accumulation in the normal organs. As a result, anatomical characteristics specific to tumor tissues could allow the preferential accumulation of the drug carrier in the tumor. As described in the introduction, such tumor-specific anatomical characteristics are characterized by vascular hyperpermeability and impaired lymphatic drainage, termed the EPR effect by others (16–18). It has been suggested that the tumor vascular permeability arises from overexpression of the vascular permeability factor/vascular endothelial growth factor gene (48–50) as well as secretion of other factors such as basic fibroblast growth factor (50), bradykinin (17, 51), nitric oxide (51, 52), peroxynitrate (53), matrix metalloproteinases (53), and prostaglandins (17) in tumor tissues. Because angiogenesis supports the abnormal tumor growth, it is conceivable that the EPR effect is universally observable in solid tumors (49, 50). Therefore, the passive targeting may be applicable to the treatment of most solid tumors. On the other hand, active targeting is proposed to selectively eradicate the tumor cells via a specific binding of targeting moieties linked to a drug vehicle with tumor-derived specific markers (22, 54). However, the tumor cells are generally located outside of microvasculatures, so that the extravasation of drug vehicles, which is a passive process governed by their molecular size and longevity in blood circulation, is a prerequisite for their specific interaction with tumor cells. There is an increasing number of evidence recently that antibody or its fragment-conjugated liposomes (immunoliposomes) and polymeric micelles (immunomicelles) improved therapeutic efficacy over nontargetable carrier systems (30, 54). Immunoliposomes and immunomicelles are attracting attention due to their high selectivity to the tumor cells, effective retention in the tumor, and internalization of drug carriers charged with many drug molecules to tumor cells. The "bystander" effect allows the drug uptake by antigen-negative cells (54). In addition, recent advances in antibody engineering have allowed for the production of humanized antibody fragments, reducing problems with immune responses against mouse antibodies (54, 55). However, the active targeted carrier systems still have several limitations regarding expensive and time-consuming procedure, problems

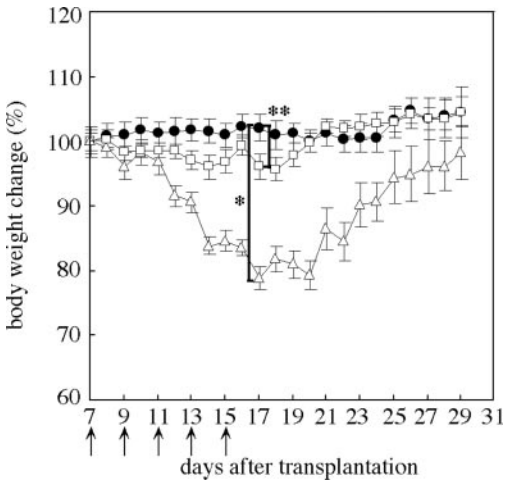

Fig. 7. Body weight change of C 26-bearing CDF1 mice (female, $n = 10$) treated with saline (●), free CDDP (△), and CDDP-incorporated micelles (□). Each drug was administered five times at 2-day intervals (*arrows*) at the dose of 4 mg/kg on a CDDP basis. The molar ratio of CDDP to block copolymer in the micelle was 27, and the dose on the injected polymer was 6.3 mg/kg. Data are expressed as mean; *bars*, ±SE. ∗ $P < 0.005$; ∗∗, not significant ($P > 0.05$).

with stability and storage, and so on (54). Hence, until recently, most of clinical applications of liposomes and polymeric micelles have been focused on the passive targeting rather than the active targeting. From the standpoint of pharmacokinetics, Marecos *et al.* (56) compared recently the tumor accumulation between nontargeted long-circulating polymers and specific monoclonal antibodies, and found that their accumulation levels were comparable after 48 h, despite a difference in early phase of their accumulation kinetics. Also, Maruyama compared long-circulating liposomes (LCLs) and transferrin-installed LCLs, and observed the same tumor accumulation profiles up to 24 h, although transferrin-installed-LCLs showed longer tumor residence than LCLs (57). In this study, we demonstrated that CDDP-incorporated micelles showed significantly enhanced tumor accumulation (Fig. 5) and high selectivity to the tumor (Table 1) in LLC-bearing mice, based on passive targeting. These properties were obviously improved when compared with the micelles reported previously from PEG-P(Asp), which showed high selectivity to the liver and spleen (15). Furthermore, the CDDP-incorporated micelles reported in this article may be superior to other formulations of CDDP such as LCL and water-soluble polymer-drug conjugates in regard to tumor-selective delivery (6, 7, 10, 58). For instance, SPI-077 (Stealth liposomal cisplatin) is an improved formulation of LCL incorporating CDDP, but exhibits much higher accumulation in the spleen than the tumor (10). The tumor-targeting efficiency of CDDP-incorporated micelles, which is defined as an accumulation ratio of the tumor to normal tissue, was found to be >1.0 for all of the main organs (Table 1), suggesting that CDDP-incorporated micelles could serve as an ideal vehicle for the passive tumor targeting *in vivo*. Because a slower elimination rate in the tumor is expected due to the impaired lymphatic system of tumor tissue (18) as mentioned previously, CDDP-incorporated micelles may localize in the interstitial space of the tumor for a longer period compared with normal tissues.

Our present results suggest a remarkably increased therapeutic efficacy of CDDP-incorporated micelles in C 26-bearing mice compared with free CDDP. Whereas no cure of primary tumors was observed for free CDDP treatment, 4 of 10 tumors were completely cured for the treatment with CDDP-incorporated micelles (Fig. 6). During the treatment, the mice treated with CDDP-incorporated micelles showed no significant body weight loss, whereas the treatment with free CDDP significantly decreased the body weight of mice to ~80% of the initial weight ($P < 0.005$; Fig. 7). The remarkable enhancement of the antitumor activity of CDDP by using drug carriers, which can cause complete regression of primary tumors, may be rare in the literature (6, 10, 11). Although the EPR effect might be a universally observable effect in solid tumors as mentioned previously, further study using various types of tumor cells combined with optimization of the drug dose and regime will be necessary. In the present study, each drug was administered five times to the mice at 2-day intervals. In such repeated drug treatment, the cytotoxicity of the drug may affect the EPR effect. Minko *et al.* (59) compared the effect of the cytotoxicity on the EPR effect between free doxorubicin (Dox) and Dox-conjugated water-soluble polymers. They found that repeated treatment with Dox-conjugated water-soluble polymers decreased vascular permeability with down-regulation of the vascular endothelial growth factor gene inside the tumor, whereas the repeated free Dox treatment increased permeability with up-regulated vascular endothelial growth factor gene expression in all parts of the tumor. This issue remains to be studied in our system. We found recently that CDDP-incorporated micelles down-regulated the genes encoding integrins and matrix metalloproteinases, which play an integral role in tumor invasion, metastasis, and angiogenesis, whereas free CDDP up-regulated them (60). The down-regulation of those genes by the micelle may contribute to its enhanced antitumor activity *in vivo* reported here.

In summary, we have demonstrated that the CDDP-incorporated micelles are a unique formulation with a sustained drug release and time-modulated decay of the carrier itself. This formulation achieved selective and significant accumulation in solid tumors according to the passive targeting manner. In *in vivo* antitumor activity assay using C 26-bearing mice, the treatment with CDDP-incorporated micelles led to complete tumor regression, which was not obtained in the treatment with free CDDP and is still rare in the literature in the development of the tumor-targeted delivery system of CDDP, indicating a potential utility of the micellar formulation incorporating CDDP in clinical tumor chemotherapy.

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