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Cisplatin-incorporated Polymeric Micelles Eliminate Nephrotoxicity, While Maintaining Antitumor Activity

Yasuo Mizumura,[1] Yasuhiro Matsumura,[1,7] Tetsuya Hamaguchi,[1] Nobuhiro Nishiyama,[2] Kazunori Kataoka,[2] Takanori Kawaguchi,[3] William J. M. Hrushesky,[4] Fuminori Moriyasu[5] and Tadao Kakizoe[6]

[1]Department of Medicine, [6]The Director, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, [2]Department of Materials Science, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, [3]2nd Department of Pathology, Fukushima Medical College, 1 Hikarigaoka, Fukushima 960-1247, [4]Stratton DVAMC/DVA Network 2, Albany Medical College of Union University, Albany, New York 12208 and [5]Fourth Department of Medicine, Tokyo Medical University, 6-7-1 Nishishinjyuku, Shinjyuku-ku, Tokyo 160-0023

cis-Diamminedichloroplatinum (II) (cisplatin, CDDP), a potent anticancer agent, was bound to the aspartic acid residues of poly(ethylene glycol)-poly(aspartic acid) (PEG-P(ASP)) block copolymer by ligand substitution reaction at the platinum atom of CDDP. The polymeric drug thus obtained was observed to form a micelle structure in aqueous medium, showing excellent water solubility. In the present study, *in vitro* and *in vivo* antitumor activity against several human tumor cell lines, toxicity and pharmacokinetic characteristics in rodents of CDDP-incorporated polymeric micelles (CDDP/m) were evaluated in comparison with those of CDDP. *In vitro*, CDDP/m exhibited 10–17% of the cytotoxicity of CDDP against human tumor cell lines. CDDP/m given by intravenous (i.v.) injection yielded higher and more sustained serum levels than CDDP. *In vivo* CDDP/m treatment resulted in higher and more sustained levels in tumor tissue than CDDP, and showed similar antitumor activity to CDDP against MKN 45 human gastric cancer xenograft. CDDP/m treatment caused much less renal damage than CDDP. These results indicate that CDDP/m treatment can reduce CDDP-induced nephrotoxicity without compromising the anticancer cytotoxicity of CDDP.

Key words: Polymeric micelles — Cisplatin — Nephrotoxicity — EPR effect — DDS

cis-Diamminedichloroplatinum (II) (cisplatin, CDDP), the most commonly used anticancer agent, consists of a central platinum atom surrounded by four ligands, two ammonias and two chlorides.[1)] A high antitumor activity results when the two chloride ligands in CDDP are bi-aquated in aqueous physiological environments; CDDP can then interact directly with DNA and display cytotoxic activity.[2, 3)] The clinical utility of CDDP is limited by significant general organ toxicity including myelosuppression,[4)] ototoxicity,[5)] gastrointestinal disturbance,[5, 6)] and especially acute nephrotoxicity.[7)] CDDP, a low-molecular-weight compound, is distributed readily into almost all tissues and intracellular compartments. CDDP traverses plasma membranes rapidly via passive diffusion or active transport, and is also rapidly cleared from blood by glomerular excretion, limiting its therapeutic availability. Injection of the maximum permissible amount of this low-molecular-weight drug to raise its therapeutic concentration and AUC (area under the curve) results in severe toxicity without significantly greater antitumor efficacy. Therefore, several novel forms of controlled release drug delivery have been designed to improve distribution and to prolong the exposure of the tumor to an effective drug concentration.

It is known that solid tumors generally possess the following pathophysiological characteristics: hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors, and also the absence of effective lymphatic drainage, preventing the efficient clearance of accumulated macromolecules. These characteristics, unique to solid tumors, are believed to be the basis of the so-called EPR effect (enhanced permeability and retention effect).[8, 9)] Moreover, macromolecules have relatively prolonged plasma half-lives because they are too large to pass through the normal vessel walls, unless they are trapped by the reticuloendothelial system.[10–12)] Therefore it is relatively easy for macromolecules to extravasate into and accumulate within tumor tissue on the basis of the EPR effect.

To make use of the EPR effect, several techniques have been developed. Yokoyama *et al.* reported that adriamycin (ADR)-containing polymeric micelles show dramatically higher antitumor activity *in vivo* than free ADR, because of highly selective delivery to solid tumor tissue through the EPR effect.[13, 14)] We have concentrated upon constructing polymeric micelles composed of poly(ethylene glycol)-poly(aspartic acid) (PEG-P(ASP)) block copolymer.[15, 16)]

[7] To whom correspondence should be addressed.
E-mail: yhmatsum@ncc.go.jp

This copolymer possesses aspartic acid residues capable of avidly binding at the platinum atom of CDDP by a ligand substitution reaction. Furthermore, PEG-P(ASP) block copolymer conjugating platinum spontaneously develops a micellar structure in aqueous solution. This structure possesses high structural stability in distilled water. In physiological saline, however, the micelles start to dissociate slowly with an induction period of approximately 10 h, synchronized with the sustained release of the platinum complex from the core. We believe that the sustained release profile of CDDP from the micelles would be advantageous for obtaining an enhanced antitumor effect *in vivo*.[17, 18)]

In the present study, we examine the potential utility of CDDP-incorporated polymeric micelle (CDDP/m) as a drug delivery system for targeting therapy with CDDP by means of *in vitro* and *in vivo* experiments.

MATERIALS AND METHODS

Chemicals CDDP was purchased from Aldrich Chemical Co., Inc. β-Benzyl L-aspartate and bis(trischloromethyl)-carbonate (triphosgene) were purchased from the Peptide Institute, Inc., Osaka, and Tokyo Kasei Kogyo Co., Ltd., Tokyo, respectively. These chemicals were used without further purification. Other chemicals were of reagent grade and were used as purchased.

Synthesis of CDDP/m, micelle-forming (PEG-P(ASP)) block copolymer conjugating CDDP PEG-P(ASP) was synthesized by a previously reported procedure.[16, 18)] The procedure for preparing CDDP/m and the method of incorporating CDDP into polymeric micelles are briefly displayed in Fig. 1.

The N-carboxy anhydride of β-benzyl L-aspartate (BLA-NCA) was synthesized by the Fuchs-Farthing method using triphosgene. Poly(ethylene glycol)-poly(β-benzyl L-aspartate) block copolymer (PEG-PBLA) was then synthesized by polymerization of BLA-NCA in dichloromethane (CH_2Cl_2) initiated by the terminal amino group of α-methyl-ω-aminopoly(ethylene glycol) (CH_3O-PEG-NH_2; M_w=5000). The degree of polymerization of BLA in the block copolymer was determined to be 40. PEG-P(ASP) block copolymer was prepared from PEG-PBLA by removal of the benzyl groups in 0.1 *N* NaOH. PEG-P(ASP) block copolymer and CDDP were dissolved in distilled water with a 1:1 molar ratio of CDDP to Asp residue. The mixture was shaken at 37°C for 72 h to obtain micelle-forming (PEG-P(ASP)) block copolymer conjugating CDDP. Purification of CDDP-incorporated micelles was carried out by ultrafiltration and confirmed by gel-permeation chromatography. Finally, polymeric micelles containing CDDP with narrow size distribution were obtained. The weight-average diameter of the particles was approximately 20 nm. Pt content was determined by using an atomic absorption spectrophotometer (Z-8000 polarized Zeeman atomic absorption spectrophotometer (Hitachi Instruments, Inc., Tokyo)). The weight ratio of CDDP in the micelles was 41.2±2.3 wt%.

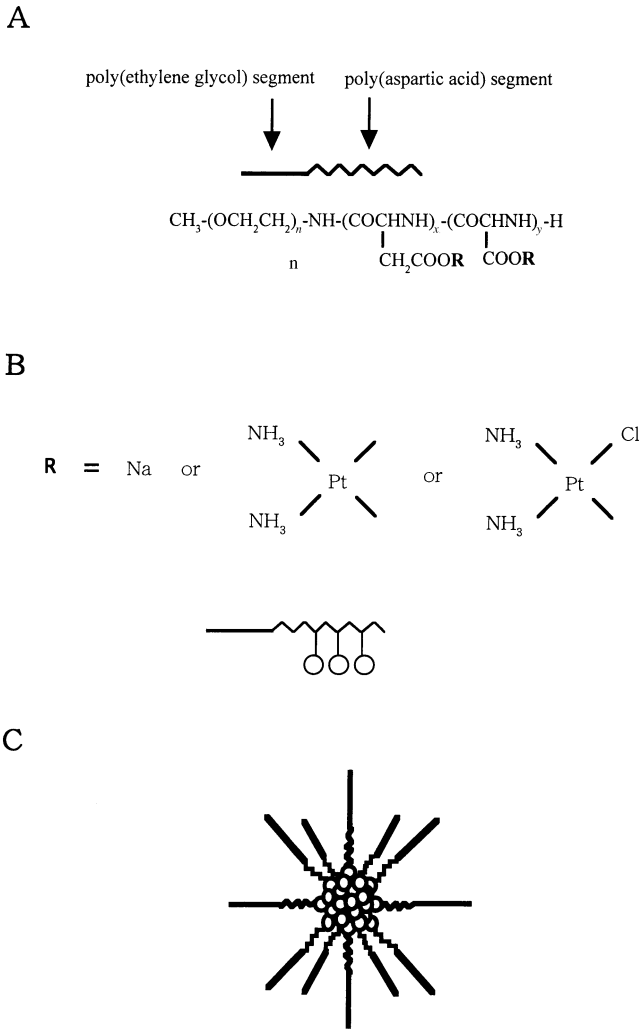

Fig. 1. Chemical structure of CDDP/m and the method of incorporating CDDP into polymeric micelles are shown. A) AB block copolymer is composed of poly(ethylene glycol) and poly(aspartic acid) segments. B) CDDP is bonded to AB-block copolymer through ligand substitution between Pt atom and Asp residues of the PEG-P(ASP) block. ○ denotes CDDP. C) polymer-metal complexes spontaneously and easily form micelles in aqueous media owing to cohesive forces between polymer and metal complexes.

***In vitro* cytotoxicity** Five colonic cancer cell lines (COLO201, COLO320, DLD-1, HT-29, LOVO), 5 gastric cancer cell lines (MKN-28, MKN-45, MKN-72, TMK-1, KATOIII), 4 breast cancer cell lines (MCF-7, 4A4, SK-

BR-3, SST, T-47-D) and 2 lung cancer cell lines (A549, PC-14) were used in this study. All the cell lines were maintained in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. For cytotoxicity analysis, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. Ten thousand cells of each cell line in 198 μl of culture medium were plated in 96-well plates 24 h prior to drug treatment. Then 2 μl of various doses of CDDP or CDDP/m was added. Cells were exposed to the indicated drug concentration in triplicate for 24, 48 or 72 h.

Plasma clearance and distribution Male C57BL/6N mice were inoculated subcutaneously with 10^6 viable Lewis lung carcinoma cells. Seven days later, tumor size had reached approximately 50–70 mm^2, measured as the product of two orthogonal diameters. Animals were i.v. injected with free CDDP (150 μg/mouse) or CDDP/m at an equivalent dose of CDDP, and sacrificed in groups of 4 at 1, 4, 8, 24 h after injection of each drug. The main organs were dissected, and blood was collected from the inferior vena cava. These samples were analyzed for total platinum content in the tissue and serum using an atomic absorption spectrometer as described previously.[19)]

***In vivo* antitumor activity** Antitumor activity was evaluated using nude mice implanted with the human gastric cancer cell line MKN-45. BALB/c *nu*/*nu* female mice (6-week-old) were inoculated at a subcutaneous (s.c.) site on the abdominal skin with one million tumor cells. Four days later, when the tumor diameter reached approximately 3 mm, the tumor-bearing mice were allocated randomly to drug treatment groups of 5 animals each. Treatment groups were as follows: free CDDP at a dosage level of 5 mg/kg; CDDP/m at an equivalent dose of CDDP; saline as control. Drugs in a volume of 0.2 ml were injected into a tail vein daily for 3 days starting on day 4 after tumor inoculation. The antitumor effect was evaluated in terms of the tumor size by measuring two orthogonal diameters ($a \times b$: a, long diameter; b, short diameter) at days 0, 5, 8, 13, 15, 18 after initial treatment.

Toxicity of free CDDP and CDDP/m

Body weight change: The toxicities of CDDP and CDDP/m were evaluated by measuring body weight changes of nude mice following i.v. administration of either saline or each of these drugs. The first injection was followed by a second 4 days later and a third 7 days later, and mice were weighed on days 1, 3, 8, 10, 11 after the first injection. Body weight measurements were stopped on day 11 because 3 toxic deaths occurred in CDDP-injected mice on day 15, the next scheduled day of body weight determination. These body weight change data were analyzed using repeated-measures two-way ANOVA.

Nephrotoxicity and pathological changes: Three groups of 5 Sprague-Dawley male rats (8-week-old, 225–250 g initial weight) were given a single injection of either CDDP (10 mg/kg) or CDDP/m at an equivalent dose, or saline.

Table I. IC_{50} Values (μM) of CDDP and CDDP/m in Various Cell Lines

	Exposure time					
	24 h		48 h		72 h	
	CDDP	CDDP/m	CDDP	CDDP/m	CDDP	CDDP/m
Colonic cancer						
Colo 201	>100	>100	>100	>100	35	>100
Colo 320	45	>100	11	72	5.0	34
DLD-1	>100	>100	11	>100	9.3	57
HT-29	>100	>100	38	>100	23	>100
Lovo	32	>100	8.0	57	3.0	19
Gastric cancer						
KATO-III	>100	>100	16	>100	9.0	49
MKN-28	>100	>100	>100	>100	7.1	41
MKN-45	>100	>100	7.1	53	5.8	30.3
MKN-74	>100	>100	>100	>100	>100	>100
TMK-1	>100	>100	31	>100	21	92
Breast cancer						
MCF-7	>100	>100	36	>100	4.6	26
4A4	37	>100	14	>100	8.7	52
T-47-D	>100	>100	>100	>100	29	>100
SST	>100	>100	62	>100	42	>100

Each cell line was treated in triplicate for 24, 48, and 72 h.
MTT assay was used for obtaining IC_{50} values.

After injection of each drug, samples of blood were taken at 7 days, and liver, kidney, small intestine, and colon were collected at 7 days. These organs were immersed in 10% formalin solution. In each blood sample, levels of blood urea nitrogen (BUN) and creatinine were measured with a Hitachi 7170.

Statistical methods Antitumor activity data and body weight change data are expressed as the mean±standard error of the mean (SE). The other data are expressed as the mean±standard deviation of the mean (SD). Comparative antitumor activity data and body weight change data were contrasted across groups using ANOVA. Other data were compared by using the two-tailed Student's *t* test. *P* values of 0.05 or less were considered statistically significant.

RESULTS

***In vitro* cytotoxicity** IC_{50} values for CDDP/m and CDDP against various cancer cell lines exposed to the indicated drug concentration for 24, 48 or 72 h are shown in Table I. IC_{50} values for CDDP/m were 6 to 10 fold higher than those of CDDP at any exposure time, indicating that the *in vitro* cytotoxic activity of CDDP/m was inferior to that of CDDP.

Plasma clearance and distribution The time courses of the levels of CDDP and CDDP/m in blood are shown in Fig. 2A. When CDDP was i.v. injected, it was cleared very rapidly and the plasma concentration of CDDP fell below 5% of the injected dose by 4 h. In contrast, CDDP/m exhibited slower clearance than CDDP. Plasma concentrations of platinum at 4 and 8 h after CDDP/m administration were 42% and 22% of the injected dose, respectively. The levels of CDDP/m attained in the solid tumors were correspondingly higher than for mice receiving CDDP (Fig. 2B). CDDP/m continued to accumulate in the tumor over the first 10 h after injection. Platinum concentration in the tumor was still approximately 25% dose/g organ at 24 h following CDDP/m administration. On the other hand, the platinum concentration was only 5% dose/g organ at 24 h after CDDP administration. However, no difference in platinum accumulation in the kidney was seen between these two drugs, as shown in Fig. 2C.

***In vivo* antitumor activity** The activity of CDDP/m or CDDP was evaluated with human gastric cancer cell line MKN-45 growing in nude mice, by i.v. injection daily for 3 days. Relative tumor growth rate of each treatment group after i.v. injection is shown in Fig. 3. Relative tumor growth rates were compared across these three treatments including the control and across time, as represented by the six measurements (days 0, 5, 8, 13, 15, 18), using a three-way analysis of variance. Overall, treatment effect was validated when control relative tumor growth rates were contrasted with the CDDP and CDDP/m treated groups (F=6.9, P=0.01). Growth over time showed the expected tumor progression in all groups (F=36, P<0.001). No interaction was found between type of treatment and observation time. These results clearly indicate highly significant treatment-dependent differences in rela-

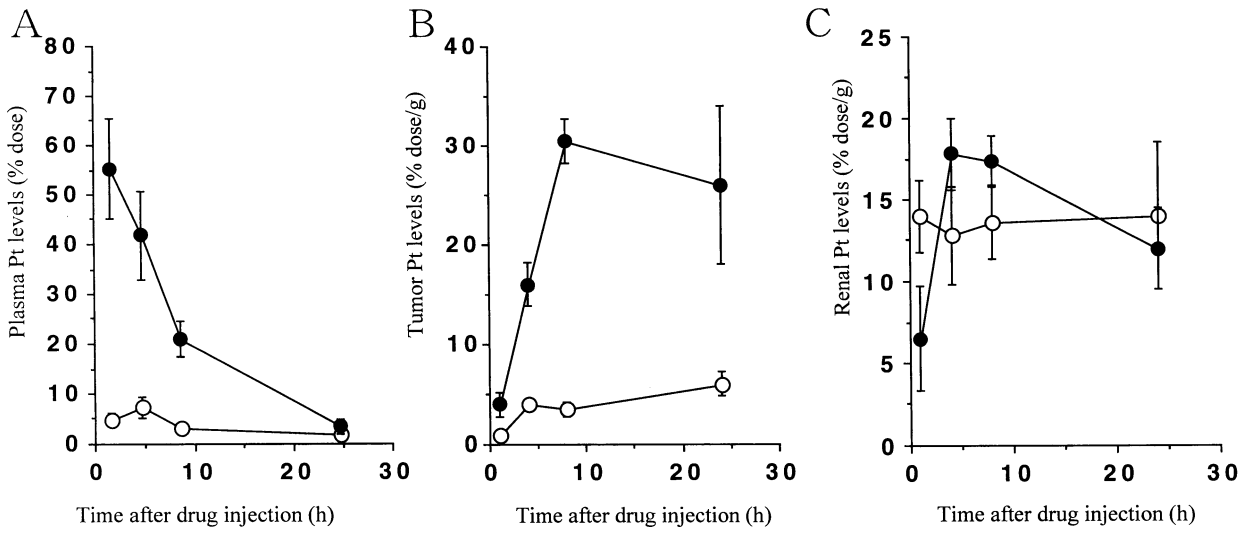

Fig. 2. Blood clearance and tumor uptake and kidney uptake of free CDDP and CDDP/m. 1×10^6 viable Lewis lung carcinoma cells were inoculated s.c. into the abdominal region of C57BL/6N mice. On day 7, CDDP was i.v. administered at the dose of 150 μg/mouse (○) and CDDP/m was i.v. administered at an equivalent dose of CDDP (●). Data are presented as mean±SE. A) Blood clearance, B) tumor platinum level, C) kidney platinum level.

tive tumor growth rate when the treatments and placebo are contrasted.

In order to determine whether the two drug preparations had different antitumor activity, a second two-way ANOVA was performed. This analysis contrasted the effects of each cisplatin treatment (CDDP versus CDDP/m) upon relative tumor growth rate over time (days 0, 5, 8, 13, 15, 18). Overall, the anticancer effects of these two compounds were equal (F=3.3, P=0.10). Tumor growth in both treatment groups over time was confirmed (F=52.4, P<0.001). There was, however, an interaction between observation time and treatment type (F=2.5, P<0.05). This interaction indicates that the results of the two treatments differed at some times of tumor measurement, but not at others. Overall, these results confirm that both CDDP and CDDP/m have anticancer activity. The anticancer activities of these compounds, in this limited study, were statistically and biologically equivalent.

Toxicity of free CDDP and CDDP/m in terms of body weight change Fig. 4 shows the body weight change of nude mice after three injections of each drug. Mean body weight for each experimental group fell throughout the experiment. Body weight loss was far greater for the CDDP-injected group than for either the control or CDDP/m-treated group (F=11, P=0.002). Body weight loss for CDDP/m-treated mice was, in fact, no greater than for mice injected with saline (control) (F=2.5, P=0.15). Comparison of body weight change after CDDP with that after CDDP/m revealed far greater toxicity of CDDP (F=10.0, P=0.01). In addition, CDDP/m caused a maximum weight loss of less than 3.3% while CDDP caused more than 5% body weight loss. Overall these results show that CDDP/m has lower toxicity, as reflected in body weight change, than CDDP.

Nephrotoxicity and pathological changes Fig. 5 shows renal function of rats 7 days after administration of each drug. Remarkable nephrotoxicity occurred, as expected, after administration of CDDP (10 mg/kg), but not CDDP/m at an equivalent dose of CDDP. BUN values 4 days after administration of saline, CDDP, and CDDP/m were 20.6±2.6, 67.3±20.5, and 24.0±4.4 mg/ml, respectively. In the case of creatinine, values of 0.24±0.06, 0.82±0.23, and 0.26±0.09 mg/ml were obtained after administration of saline, CDDP, and CDDP/m, respectively. BUN and creatinine values rose after administration of CDDP, compared to the control or CDDP/m (BUN: P<0.001, P<0.001, respectively, creatinine: P<0.001, P<0.001 respectively). CDDP/m caused no significant elevation of either BUN or creatinine relative to saline treatment. Renal pathological change revealed that tubular cell

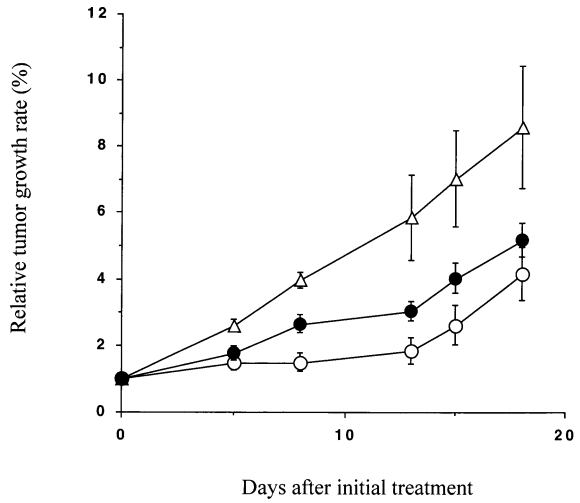

Fig. 3. Changes in relative tumor (MKN-45) growth rate based on tumor diameter ($a \times b$: a, long diameter; b, short diameter) in abdominal skin in nude mice after treatment with CDDP or CDDP/m. Relative tumor-growth rates were measured in each treatment group: CDDP at 5 mg/kg (○), CDDP/m (●) at an equivalent amount to CDDP, or saline (△), given by i.v. injection daily for 3 days starting on day 4 after tumor inoculation. Point, mean values; bars, ±SE.

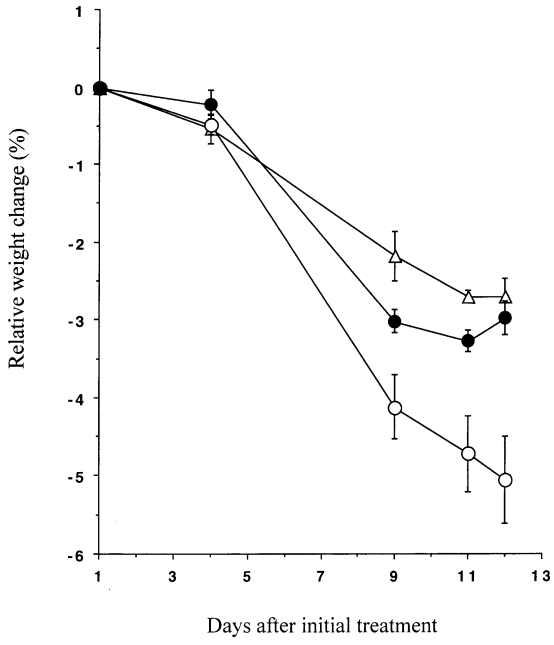

Fig. 4. The toxicities of CDDP (5 mg/kg) (○) and CDDP/m (5 mg/kg) (●) were evaluated by measuring body weight changes. Nude mice (n=5) were given either saline (△) or one of these drugs. The first injection was followed by a second 4 days later and a third 7 days later, and mice were weighed on days 1, 3, 8, 10, 11 after the first injection. Mean body weights for each experimental group fell throughout the experiment.

degeneration as indicated by tubular dilatation with flattening of the lining cells of tubular epithelium and tubular regeneration as indicated by enlarged nuclei occurred only among rats receiving CDDP (Fig. 6). The small intestine showed extensive degeneration of mucous epithelium in rats treated with CDDP (Fig. 7). On the other hand, there was no pathological change at all in major organs, such as liver, lung, heart, and spleen, of rats treated with CDDP/m.

DISCUSSION

CDDP is one of the most broadly active and most widely used anticancer agents for the treatment of common solid tumors. However CDDP has severe toxic effects on normal cells of the gut, bone marrow,[4] nerves and the renal tubular epithelium.[20–22]

To overcome those problems, several platinum-polymer preparations have been investigated, including polylactic acid microcapsules,[23] and microspheres consisting of L-lactic acid,[24] dextran,[25] and HPMA copolymer.[26] Most preparations have failed to demonstrate meaningful benefit *in vivo*. Their failure may be due to structural instability in blood and the inability to incorporate enough CDDP into the copolymer.

Ringsdorf *et al.*[27] proposed that AB block copolymer-drug conjugates might form micelle structures that could improve drug solubility. An AB-type block copolymer forms such a structure because of its amphiphilic character. The polymer drug-binding sites in the polymer-drug conjugates form the hydrophobic core of the micelles in aqueous media. This is surrounded by an outer hydrated shell, which is expected to bring about high water solubility by inhibiting intermicellar aggregation. These polymeric micelles have a narrow, unimodal diameter distribution in the range from 20 to 60 nm. The size and narrow size distribution of micelles are expected to optimize the rate of accumulation to the tumor site while minimizing renal excretion. The micelles have long half-lives in the bloodstream, permitting large amounts of the micelles to reach the tumor site. The utility of polymeric micelles in cancer chemotherapy has been demonstrated in the case of ADR-incorporated polymeric micelles by Yokoyama *et al.*[14] ADR-incorporated polymeric micelles (ADR/m) decreased the toxicity of ADR significantly in terms of body weight change and yet exhibited superior *in vivo* antitumor activity against several solid tumors in comparison with free ADR. In this study, using the same rationale, we have found that the incorporation of CDDP into polymeric micelles affords several advantages over CDDP alone.

In the *in vitro* study, the cytotoxicity of CDDP/m against various cancer cell lines was negligible after 24 h of incubation. Initially we had speculated that CDDP/m would gradually release platinum, so that long times might be necessary for CDDP/m to achieve adequate cytotoxicity. However, CDDP/m appeared to possess inadequate cytotoxicity in comparison with CDDP even after 48 or 72 h of incubation with all the cells used in this study (Table I). We hypothesize that the decrease of cytotoxicity of CDDP/m is due to the extreme *in vitro* stability of the micellar structure, so that CDDP/m may not release free CDDP in a culture dish. Nishiyama *et al.*[18] reported that CDDP/m began to dissociate after an induction period of approximately 10 h, accompanied with sustained release of platinum complex from the micelles, in physiological saline at 37°C. In addition, it was reported that the release rates of platinum complex from CDDP/m in physiological saline at 24 h and 72 h were approximately 50% and 75%, respectively. CDDP/m was quite stable for more than 80 h in distilled water. Therefore, a much longer *in vitro* expo-

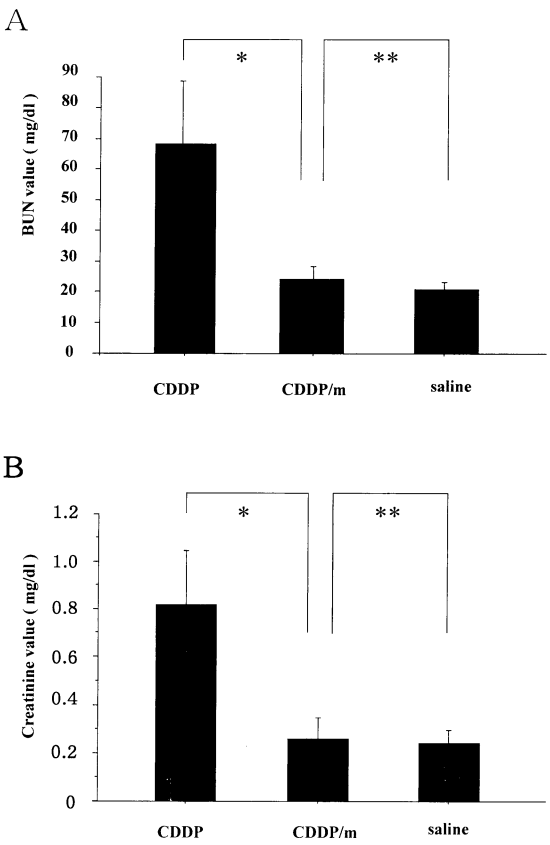

Fig. 5. CDDP/m-induced nephrotoxicity, compared with CDDP. Renal function of rats 7 days after administration of saline, CDDP at a dose of 10 mg/kg or CDDP/m in an equivalent amount to CDDP was examined. A) BUN value, B) creatinine value. Data are presented as mean±SD. * $P<0.001$, ** not significant.

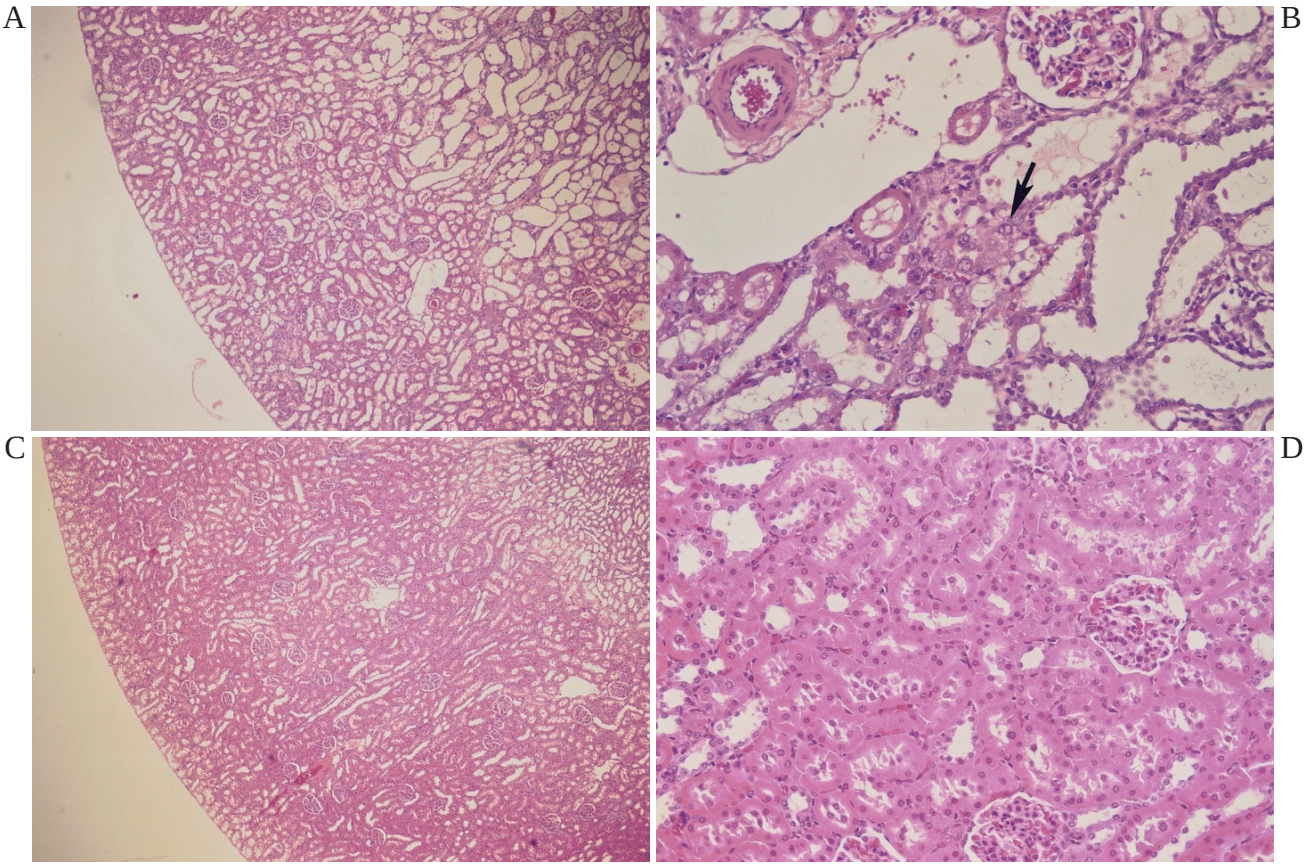

Fig. 6. Histological appearance of renal tubules in rats 7 days after i.v. administration of free CDDP at 10 mg/kg (A, ×4 and B, ×20) and CDDP/m (in an equivalent dose to CDDP) (C, ×4 and D, ×20). In an animal given CDDP, widespread tubular degeneration with flattening of the lining cells of the renal cortex was seen (A). Tubular regeneration of the renal cortex, as indicated by enlarged cells with large nuclei (arrow), also appeared (B). An animal given CDDP/m showed no distinct pathological change in renal tubuli.

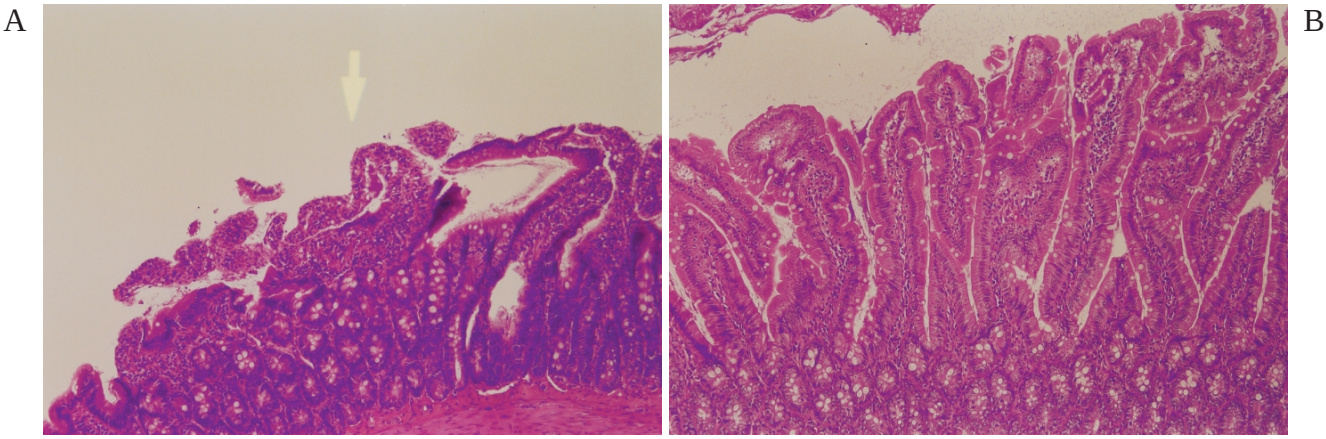

Fig. 7. Histological appearance of intestinal epithelium in rats 7 days after i.v. administration of free CDDP (A) and CDDP/m (B). A: the villi of the intestinal mucosal surface were irregularly denuded (arrow). B: there was no pathological change in the intestinal mucosa.

sure may be necessary for CDDP/m to exhibit cytotoxicity to cells in culture, because the cytotoxicity of CDDP/m depends on the availability of free platinum via the cleavage of carboxylic ligands of the platinum complex.

In the *in vivo* study, CDDP/m exhibited dramatically longer circulation times than CDDP. These pharmacokinetics may also reflect micelle stability in the blood stream. This means that the micelle carrier system can, in principle, deliver higher amounts of CDDP to tumor tissue. In addition, CDDP/m also diffuses out of tumor vessels more effectively due to the EPR effect.[8, 9] Micelles accumulated in the tumor tissue are apparently degraded, and free CDDP is ultimately released from the multimolecular micellar structure in adequate concentrations to kill nearby tumor cells. These pharmacodynamic effects help to explain how CDDP/m exhibits an antitumor efficacy similar to that of CDDP *in vivo* (Fig. 3), in spite of its profoundly weaker antitumor activity *in vitro* (Table I). A more detailed pharmacological analysis is under way.

The most remarkable difference between CDDP/m and CDDP is the total absence of nephrotoxicity of CDDP/m following i.v. injection of equivalent doses of each drug. Pathological and biochemical studies revealed that serious nephrotoxicity occurred when rats were given 10 mg/kg of CDDP, but not when CDDP/m was given at an equivalent platinum dose. CDDP/m exhibited a much higher AUC than CDDP, but nevertheless, accumulation of Pt in the kidney at 24 h after i.v. injection was identical for each drug. Therefore the remarkable difference of nephrotoxicity between CDDP and CDDP/m could not be explained in terms of the total accumulation of platinum in the kidney.

Although total CDDP accumulation in kidney tissue is not different, the dynamics of that accumulation during the first 24 h after i.v. administration is quite different. This can be inferred from the data in Fig. 2A. After CDDP/m administration, plasma concentrations of platinum remained elevated for more than 10 h. An identical dose of CDDP is, however, cleared almost completely from plasma within minutes. Since >90% of the CDDP eliminated from the organism is cleared in the urine, these data mean that very large differences in urinary tubular concentrations of platinum occur in the first few hours after administration of these two CDDP preparations.

It is well known that peak urinary CDDP concentration correlates with nephrotoxicity much better than total renal platinum concentration.[28] This is because gradual CDDP appearance allows the tubular sulfhydril defenses to cope more successfully with the nephrotoxin. The success of saline/mannitol hydration protocols and multiday CDDP regimens for diminishing cisplatin nephrotoxicity are based upon this predictable relationship between peak tubular urinary cisplatin concentration and resulting nephrotoxicity.[29] In fact, any strategy that allows gradual rather than sudden proximal and distal renal tubular CDDP accumulation diminishes the nephrotoxicity. CDDP/m is one such successful strategy that apparently requires no concomitant medications or hydration.

In conclusion, CDDP/m eliminated CDDP toxicities without attenuating the antitumor effect of CDDP. These results suggest that CDDP/m is a promising candidate for clinical trial for the treatment of solid tumors.

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REFERENCES

1) Rozencwing, M., Von Hoff, D. D. and Slovik, M. *Cis*-diamminedichloroplatinum (II), a new anticancer drug. *Ann. Intern. Med.*, **86**, 803–812 (1977).
2) Van der Veer, J. L. and Reedijk, J. Investigating antitumor drug mechanisms. *Chem. Br.*, **24**, 775–780 (1988).
3) Zwelling, L. A. and Kohn, K. W. Mechanism of action of *cis*-dichlorodiammineplatinum (II). *Cancer Treat. Rep.*, **63**, 1439–1444 (1979).
4) Bruckner, H. W., Cohen, C. J., Wallach, R. C., Kabakow, B., Deppe, G., Greenspan, E. M., Gusberg, S. B. and Holland, J. F. Treatment of advanced ovarian cancer with *cis*-dichlorodiammineplatinum (II): poor-risk patients with intensive prior therapy. *Cancer Treat. Rep.*, **62**, 555–558 (1978).
5) Von Hoff, D. D., Schilisky, R., Reichert, C. M., Reddick, R. L., Rozencweig, M., Young, R. C. and Muggia, F. M. Toxic effect of *cis*-dichlorodiammineplatinum (II) in man. *Cancer Treat. Rep.*, **63**, 1527–1534 (1979).
6) Prestayko, A. W., D'Aoust, J. C., Issel, B. F. and Crooke, S. T. Cisplatin (*cis*-diamminedichloroplatinum (II)). *Cancer Treat. Rev.*, **6**, 17–39 (1979).
7) Madias, N. E. and Harrington, J. T. Platinum nephrotoxicity. *Am. J. Med.*, **65**, 307–314 (1978).
8) Matsumura, Y. and Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS. *Cancer Res.*, **46**, 6387–6392 (1986).
9) Maeda, H. and Matsumura, Y. Tumoritropic and lymphotropic principles of macromolecular drugs. *Crit. Rev. Ther. Drug Carrier Syst.*, **6**, 193–210 (1989).
10) Matsumura, Y., Maruo, K., Kimura, M., Yamamoto, T.,

Konno, T. and Maeda, H. Kinin-generating cascade in advanced cancer patients and *in vitro* study. *Jpn. J. Cancer Res.*, **82**, 732–741 (1991).
11) Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other diseases. *Nat. Med.*, **1**, 27–31 (1995).
12) Dvorak, H. F., Nagy, J. A., Dvorak, J. T. and Dvorak, A. M. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Am. J. Pathol.*, **133**, 95–109 (1988).
13) Yokoyama, M., Miyauchi, M., Yamada, N., Okano, T., Sakurai, Y., Kataoka, K. and Inoue, S. Characterization and anticancer activity of micelle-forming polymeric antitumor drug adriamicin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer. *Cancer Res.*, **50**, 1693–1700 (1990).
14) Yokoyama, M., Okano, T., Sakurai, Y., Ekimoto, H., Shibazaki, C. and Kataoka, K. Toxicity and antitumor activity against solid tumors of micelle-forming polymeric antitumor drug and its extremely long circulation in blood. *Cancer Res.*, **51**, 3229–3236 (1991).
15) Yokoyama, M., Inoue, S., Kataoka, K., Yui, N., Okano, T. and Sakurai, Y. Molecular design for missile drug: synthesis of adriamycin conjugated with immunoglobulin G using poly(ethylene glycol)-block-poly(aspartic acid) as intermediate carrier. *Makromol. Chem.*, **190**, 2041–2054 (1989).
16) Yokoyama, M., Miyauchi, M., Yamada, N., Okano, T., Sakurai, Y., Kataoka, K. and Inoue, S. Polymeric micelles as novel drug carrier: adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer. *J. Controlled Release*, **11**, 269–278 (1990).
17) Yokoyama, M., Okano, T., Sakurai, Y., Suwa, S. and Kataoka, K. Introduction of cisplatin into micelle. *J. Controlled Release*, **39**, 351–356 (1996).
18) Nishiyama, N., Yokoyama, M., Aoyagi, T., Okano, T., Sakurai, Y. and Kataoka, K. Preparation and characterization of self-assembled polymer-metal complex micelle from *cis*-dichlorodiammineplatinum (II) and poly(ethylene glycol)-poly(α,β-aspartic acid) block copolymer in an aqueous medium. *Langmuir*, **15**, 377–383 (1999).
19) Newman, M. S., Colbern, G. T., Working, P. K., Engbers, C. and Amantea, M. A. Comparative pharmacokinetics, tissue distribution, and therapeutic effectiveness of cisplatin encapsulated in long circulating, pegylated liposomes (SPI-077) in tumor-bearing mice. *Cancer Chemother. Pharmacol.*, **43**, 1–7 (1999).
20) Singh, G. A possible cellular mechanism of cisplatin-induced nephrotoxicity. *Toxicology*, **58**, 71–80 (1989).
21) Fillastre, J. P. and Raguenez-Viotte, G. Cisplatin nephrotoxicity. *Toxicol. Lett.*, **46**, 163–175 (1989).
22) Ban, M., Hettich, D. and Huguet, N. Nephrotoxicity mechanism of *cis*-platinum (II) diamine dichloride in mice. *Toxicol. Lett.*, **71**, 161–168 (1994).
23) Araki, H., Tani, T. and Kodama, M. Antitumor effect of cisplatin incorporated into polylactic acid microcapsules. *Artif. Organs*, **23**, 161–168 (1999).
24) Kumagai, S., Sugiyama, T., Nishida, T., Ushijima, K. and Yakushiji, M. Improvement of intraperitoneal chemotherapy for rat ovarian cancer using cisplatin-containing microspheres. *Jpn. J. Cancer Res.*, **87**, 412–417 (1996).
25) Ohya, Y., Masunaga, T., Baba, T. and Ouchi, T. Synthesis and cytotoxic activity of dextran-immobilizing platinum (II) complex through chelate-type coordination bond. *JMS Pure Appl. Chem.*, **A33**, 1005–1016 (1996).
26) Gianasi, E., Wasil, M., Evagorou, E. G., Keddle, A., Wilson, G. and Duncan, R. HPMA copolymer platinates as novel antitumour agents: *in vitro* properties, pharmacokinetics and antitumour activity *in vivo*. *Eur. J. Cancer*, **35**, 994–1002 (1999).
27) Bader, H., Ringsdorf, H. and Schmidt, B. Water-soluble polymers in medicine. *Angew. Makromol. Chem.*, **123/124**, 457–485 (1984).
28) Levi, F. A., Hrushesky, W. J. M., Borch, R. F., Pleasants, M. E., Kennedy, B. J. and Halberg, F. Cisplatin urinary pharmacokinetics and nephrotoxicity: a common circadian mechanism. *Cancer Treat. Rep.*, **66**, 1933–1938 (1982).
29) Levi, F. A., Hrushesky, W. J. M., Halberg, F., Langerin, T. R., Haus, E. and Kennedy, B. J. Lethal nephrotoxicity and hematologic toxicity of *cis*-diamminedichloroplatinum ameliorated by optimal circadian timing and hydration. *Eur. J. Cancer Oncol.*, **18**, 471–477 (1982).