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Percutaneous intratumoral injection of cisplatin microspheres in tumor-bearing rats to diminish acute nephrotoxicity

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Poly(D,L-lactide) microspheres loaded with cisplatin (PLA-CDDP MS) were prepared by a solvent evaporation technique for direct intratumoral injection. The microspheres, 50–100 μm, containing 40.04% of cisplatin produce sustained release *in vitro*. PLA-CDDP MS (6 mg/kg body weight of cisplatin) suspensions were injected intratumorally into mammary tumors in rats. Cisplatin solution (6 mg/kg body weight) was injected either intratumorally or intraperitoneally in two groups. After treatments, the tumor size decreased in each of the groups as a function of time. Sixteen days post-injection, the tumors had either disappeared or significantly shrunk. PLA-CDDP MS had a similar antitumor effect compared with cisplatin aqueous solution. Blood urea nitrogen, serum creatinine and histopathology examinations revealed that the renal toxicity in the PLA-CDDP MS group was significantly less than in the control groups. These results indicate that intratumoral injection of PLA-CDDP MS maintains anticancer potency and reduces acute renal toxicity.

Key words: Cisplatin, intratumoral injection, microspheres.

Introduction

The prognosis for primary and metastatic liver cancer remains poor. In those patients who are unsuitable for surgery, current palliative treatments have little to offer in most cases and have an associated morbidity and mortality.[1] Transcatheter arterial embolization (TAE) is a widely used and effective means of treating hepatoma. However, it is almost impossible to achieve complete necrosis of the tumor by embolization of the hepatic artery alone.[2] This therapy cannot be performed in some cases for technical reasons, e.g. iodine allergy, severe liver dysfunction or undetectability by angiography. Furthermore, TAE can possibly cause further damage to the liver.[3–6]

Interstitial therapeutic methods are another option which have several advantages, they are effective at inducing necrosis of focal hepatic tumors by relatively non-invasive approaches, morbidity and mortality rates are low, and hospitalization times are acceptably short. However, none of these techniques, such as cryotherapy, alcohol injection or interstitial radiotherapy, is well established.[1]

Cisplatin, a cell-cycle non-specific antineoplastic drug, has been shown to be effective in the management of various human carcinomas. Nevertheless, cisplatin is a highly toxic drug with a low therapeutic index and a therapeutic response is not likely to occur without some evidence of toxicity. To achieve a high concentration of cisplatin in tumors and reduced adverse effects, we explored a new approach to drug delivery. This paper reports on the experimental evaluation of a new method for interstitial cisplatin administration. The drug was entrapped in poly(D,L-lactide) microspheres, which were then injected directly into intramuscular tumors. Efficacy and toxicity results are reported.

Materials and methods

Materials

Poly(D,L-lactide) (molecular weight 15 000–25 000 Da) was obtained from Polysciences (Warrington, PA). Cisplatin and polyvinyl alcohol (PVA) (molecular weight 30 000–70 000) were purchased from Sigma (St Louis, MO). Cisplatin for injection (Platinol) was purchased from Bristol (Syracuse NY).

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Champure™ methylene chloride, supplied by Curtin Matheson Scientific (Houston, TX), was used without further purification. *N,N*-Dimethylformamide (DMF) was supplied by Fisher Scientific (Fair Lawn, NJ). Female Fischer 344 rats (180 g body weight) were obtained from Harlan (Indianapolis, IN). Mammary tumor cell line (13762 NF) was supplied by the Department of Veterinary Medicine, University of Texas MD Anderson Cancer Center, Houston, TX.

Preparation of PLA-CDDP MS

PLA-CDDP MS were prepared by an emulsion solvent evaporation procedure in a sterile hood. Cisplatin crystals were ground manually to a fine powder with a mortar and pestle. Cisplatin fine powder (0.9 g) was suspended in a solution of PLA (1.0 g) in methylene chloride (13 ml) and sonicated for 5 min. The organic phase was then slowly injected through a 21 G needle into a beaker containing saline solution, PVA (3%, w/v, pH 7.0) and cisplatin (0.5%, w/v) which was being stirred at 350 r.p.m. After administration of the organic phase, the solution was stirred continuously for an additional 5–6 h at room temperature (25°C) to evaporate the methylene chloride. Microspheres were collected by filtration through a nylon filter (20 μm mesh), washed with 1000 ml of sterile water and air dried overnight in the hood. The microspheres were fractionated using mechanical sieves and the 50–100 μm fraction was collected into a sterile vial. The vial containing microspheres was kept in a vacuum oven (30 mm in Hg at room temperature) for 3 days to completely evaporate any residual methylene chloride.

Microscopic examination

The microspheres were diluted to varying densities in water for scanning electron microscopy, 100 μl of each sample was placed onto a 0.1 μm Nuclepore membrane and air dried. The dried filters were mounted onto stubs and sputter-coated with 200 Å gold–palladium, 80:20, in a Hummer VI (Technics, Springfield, VA) and examined in a Hitachi Model S520 scanning electron microscope.

Analysis of cisplatin content

Microspheres (50.1 mg, 50–100 μm fraction) from each batch were dissolved in DMF (20 ml) and the solution was diluted with DMF to make different concentrations. The amount of cisplatin in the resulting solution was determined spectrophotometrically using a Perkin Elmer model 55 UV spectrophotometer (Coleman Instruments Division, Oak Brook, IL) at 310 nm. A standard curve was produced by dissolving a known amount of cisplatin in DMF. The experiment was performed in triplicate. The drug content was calculated as a percentage of cisplatin in total weight of the microspheres.

Determination of the *in vitro* release rate of cisplatin microspheres

PLA-CDDP microspheres (30.12 mg, 50–100 μm fraction) were incubated in phosphate-buffered saline (PBS, pH 7.4, 15 ml) in a centrifugation tube at 37°C. At various times (up to 96 h), tubes were mixed well and centrifuged at 2500 r.p.m. for 10 min. The supernatant was collected and the concentration of cisplatin was determined by UV spectrometry. The supernatant was returned to the tubes after each measurement. Cisplatin fine powder (12.06 mg) was incubated in 15 ml of PBS and measured by the same method. The measurements were run in triplicate and the amount of cisplatin released was expressed as a percentage of the total drug load.

Animal studies

All experimentation involving animals was approved by the Institutional Animal Care and Use Committee of our Institution. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current US Department of Agriculture, Department of Health and Human Service, and National Institutes of Health regulations and standards.

Mammary tumor cells (13762 NF) were inoculated into the right thigh region of 54 female Fischer 344 rats (i.m., 1×10^5 cells/rat). When the tumor size reached 1.5–2.5 cm in diameter (13 days), the tumor was measured in two dimensions. The rats were randomly divided into five groups: PLA-CDDP MS, CDDP intratumoral (i.t.), CDDP intraperitoneal (i.p.), saline intratumoral and Omnipaque 300 intratumoral. In the PLA-CDDP MS group, microspheres (6 mg/kg body weight of cisplatin) were suspended in 0.3 ml of radiographic contrast medium Omnipaque 300 (Sanofi Winthrop Pharmaceuticals, New

York, NY) before use. In control group I (CDDP i.t.) and control group II (CDDP i.p.), fresh cisplatin (6 mg/kg body weight). Omnipaque 300 solution (concentration 1 mg/ml) was injected intratumorally or intraperitoneally, respectively. After anesthetizing the rats with sodium pentobarbital (6 mg/100 g body weight, i.p.), the PLA-CDDP MS suspension was directly injected into the center of the tumor through 1.0 ml syringes (22 G needles). The fresh cisplatin solution was injected intratumorally or intraperitoneally through 22 G needles. Four rats in each group were sacrificed at day 2, 5, 7 and 16 post-injection. Blood samples were collected for BUN and creatinine assays after sacrificing. To evaluate the anticancer effect of cisplatin, two dimensions of the tumors were measured on day 2, 5, 7 and 16, and the volumes of the tumors were calculated as a percentage of the original volume. The formula used to calculate tumor volume was: $V=4/3\ \pi a^2 c$, where a is the short axis; c is the long axis. Autopsies were performed, and the kidneys were removed, weighed and fixed in 10% buffered formalin for histopathologic exams. In two blank control groups (total of six rats), three rats received intratumoral injection of saline (saline group, 0.3 ml); the others were injected intratumorally by Omnipaque 300 (Omnipaque group, 0.3 ml). The tumor size was measured before and after injection. Student's *t*-test was used for statistical evaluation.

Results

Morphology, drug content and *in vitro* release pattern of PLA-CDDP MS

Scanning electron microscopic observations showed that the microspheres were spherical in shape with a narrow size range. Crystals were found entrapped in the particle and are believed to be cisplatin (Figure 1). Approximately 65% of the particles in each batch of PLA-CDDP MS were 50–100 μm in diameter. Cisplatin content was 40.04% (w/w). As shown in Figure 2, 100% of cisplatin fine powder dissolved in PBS within 3 h, while only 16.24% of cisplatin was released from the microspheres in the same time, and 95.28% was released in 96 h.

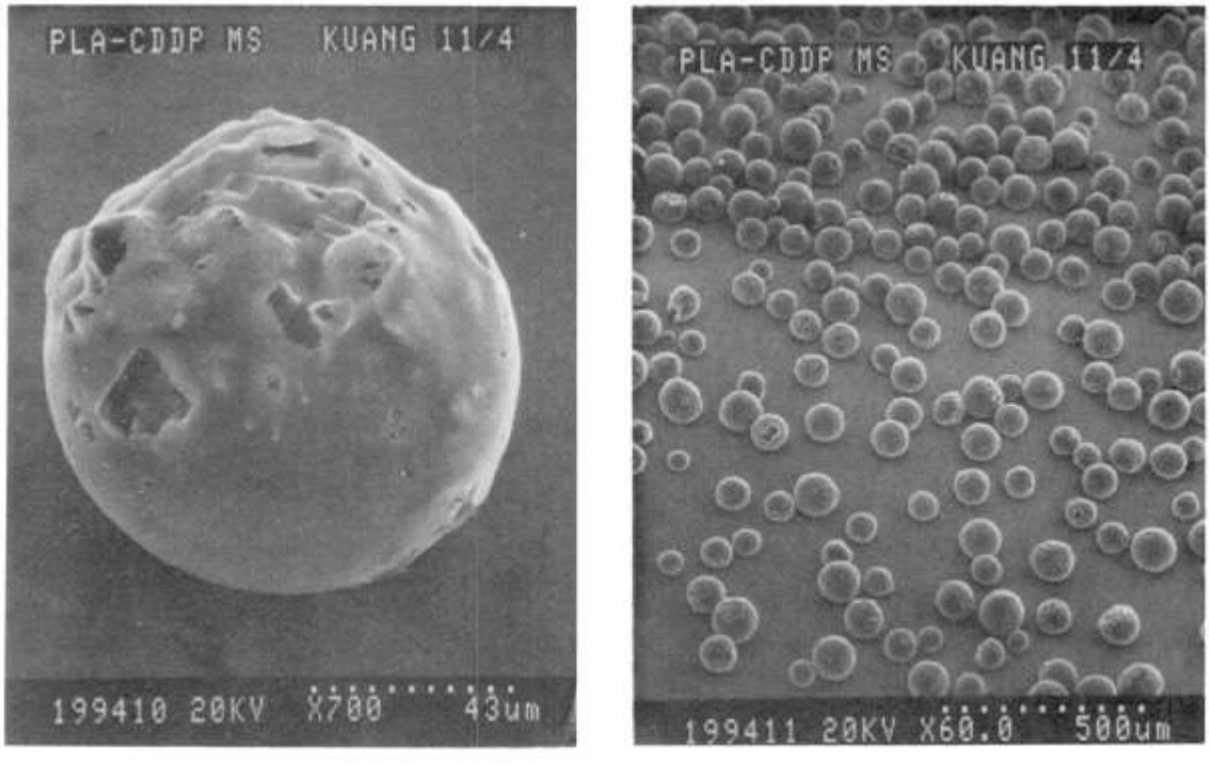

Figure 1. Scanning electron micrograph of PLA-CDDP MS (size: 50–100 μm), 40.04% (w/w) cisplatin loading.

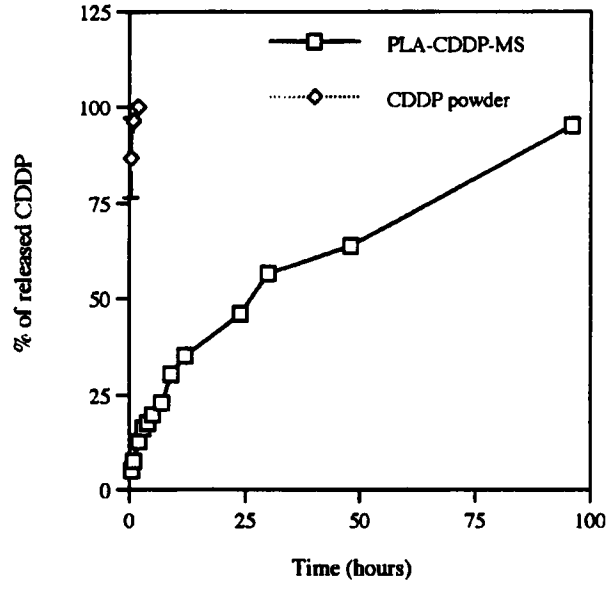

Figure 2. *In vitro* release study (37°C in PBS), PLA-CDDP MS is released significantly slower compared to cisplatin powder.

Injection technique

The 50–100 μm sized PLA-CDDP MS suspended well in Omnipaque 300 and the suspension was easily injected through a 22 G needle. Saline suspension was not suitable for injection because the microspheres blocked the needles. For a 180 g rat, the exact dose of microspheres was 2.7 mg. After the suspension was injected, some remnants of microspheres could be seen in the 'dead space' of the needle and the syringe. To prevent this from resulting in a dose reduction of the microspheres, an additional 0.8 mg of microspheres was added to fill the 'dead space'. In cisplatin control groups, the volume (1.08 ml) of the cisplatin solution was relatively large for 2.0 cm tumors. After injection, some solution leaking out from the tumor to the skin was observed. To prevent further leaking, the puncture point was pressed after removing the needle.

Evaluation of cisplatin anticancer effect

Thirteen days after inoculation of the tumor cells, the right thigh of each of the 54 rats contained a single, solid, oval or spherical, intramuscular tumor mass that was 1.5–2.5 cm in diameter. In blank control groups, tumors continuously grew after injection of saline or Omnipaque. The tumor size in both groups reached about 2.8 × 4.0 cm by day 7.

Most of the rats tolerated the cisplatin treatment. Two rats that received i.t. CDDP solution died on day 15 and one rat that received i.p. CDDP solution died on day 6. The anticancer effect of the treatments was determined either by the tumor volume or by histopathologic examinations. Two days after treatment, the slight increase of tumor volume was noted in the MS, CDDP i.t. and CDDP i.p. groups, the tumor volume continuously grew 2 days after injection. Five days later, the tumors began to shrink as a function of time in all groups. On day 16, most of the tumors had disappeared or significantly shrunk in all three groups. Small residue masses (diameter from 0.3 × 0.2 to 0.6 × 0.4 cm) were found in three of four rats of the MS group. Residue masses (0.3 × 0.3 to 0.6 × 0.4 cm) were found in two rats of the CDDP i.t. group. The sizes of the tumor residue masses found in three of four rats of the CDDP i.p. group were 0.3 × 0.2 to 0.4 × 0.3 cm. Statistically, there was no significant difference in tumor volume among the three groups on 2, 5, 7 and 16 days after treatment ($p > 0.05$). The histopathologic examinations showed that either no viable tumor existed or all the residue masses were necrotic. Figure 3 shows the tumor volume change after treatments of three groups at different times. Necropsy was performed on days 2, 5, 7 and 16.

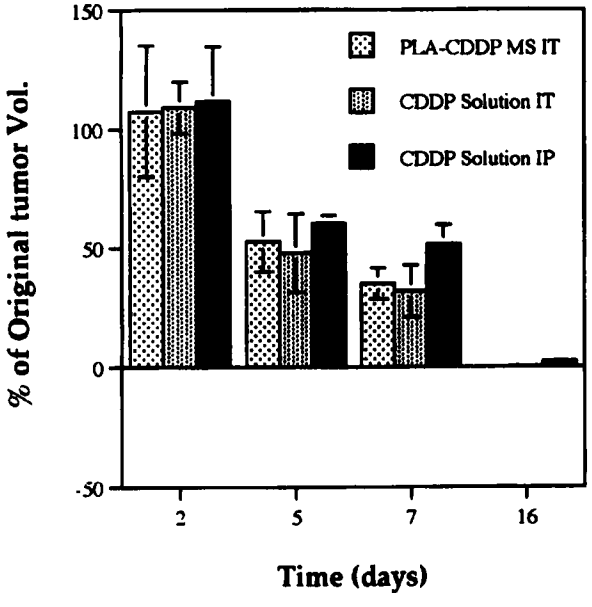

Figure 3. Five days after cisplatin treatments (6 mg/kg), tumors had shrunk as a function of time in the three groups of rats. Sixteen days after injection, tumour volumes were 0.035–0.307% of the oringinal tumour in PLA-CDDP MS, and CDDP i.t. groups, respectively. In CDDP i.p., the mean tumour volume was decreased too. There were no statistical differences among the three groups ($p > 0.05$).

No gross metastases were found in the lungs, liver or viscera in any group. No liquefaction necrosis was seen in any of the residual masses.

Renal toxicity

Following injection of PLA-CDDP MS, the highest mean BUN and creatinine levels of the rats during the 16 days follow-up were 19.58 ± 1.9 and 0.6 mg/dl, respectively. The highest mean BUN and creatinine levels in the rats in the CDDP i.p. group were 246.42 ± 75.07 and 7.03 ± 2.79 mg/dl, respectively. These peaks occurred on day 5 and were significantly greater than in the MS group. The BUN and creatinine changes in the CDDP i.p. group were similar to the CDDP i.t. group, they were up to 209.67 ± 20.13 mg/kg and 5.77 ± 0.31 mg/dl, respectively (Figures 4 and 5).

The mean kidney wet weights from the CDDP i.t. and CDDP i.p. group rats were significantly heavier than that from the MS group animals on day 2 ($p = 0.023$) and day 16 ($p = 0.034$) (Figure 6).

Histopathologic findings in the kidneys were mainly tubular damage including: tubular epithelial cell necrosis; enlarged epithelium; variability in shape, size and polarity; obscure lumina; and cystic dilation. Other findings were congestion of interstitial capillaries in the medulla and lymphocytic interstitial nephritis. Definition of scores and terminology ranking were used to differentiate the seriousness of renal damage in three animal groups. Histopathologic examination reveal acute renal toxicity of the experimental rats during the 16 days

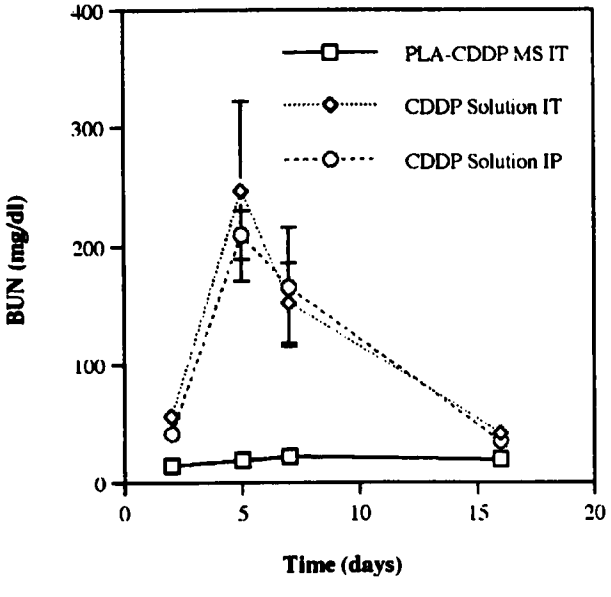

Figure 4. BUN profile after treatment (6 mg/kg). BUN significantly increased up to 246.42 ± 75.07 mg/dl in the CDDP i.t. group and 209.67 ± 20.13 mg/dl in the CDDP i.p. group, respectively. The highest mean BUN in the PLA-CDDP MS group during 16 days was 19.58 ± 1.9 mg/dl.

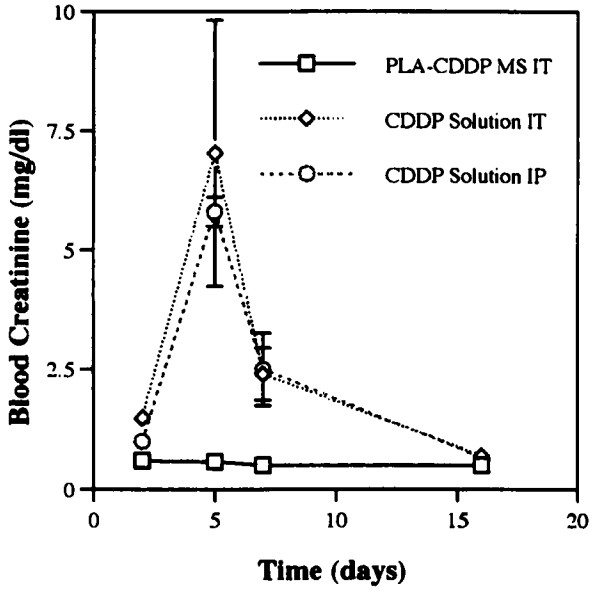

Figure 5. Serum creatinine (Cr) profile after treatment (6 mg/kg). Cr significantly increased up to 7.03 ± 2.79 mg/dl in the CDDP i.t. group and 5.77 ± 0.31 mg/dl in the CDDP i.p. group, respectively. The highest mean Cr in the PLA-CDDP MS group during 16 days was 0.6 ± 0.00 mg/dl.

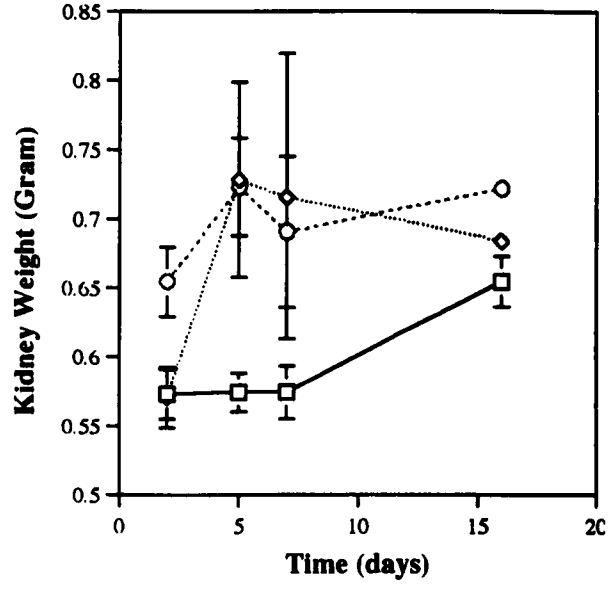

Figure 6. Weight change of the rat kidneys after cisplatin treatment (6 mg/kg). The significant difference between the CDDP i.t. (◇), CDDP i.p. (○) and PLA-CDDP MS (□) groups was on day 5.

Table 1. Rat kidney histopathology and score

	2 days	5 days	7 days	16 days
PLA-CDDP MS i.t.	0	+	+	+ +
	0	0	+ +	+ +
	+	+ +	+ +	+ + +
	0	+	+ +	+
CDDP solution i.t.	+	+ + + +	+ + + +	+ + + +
	+ + +	+ + + +	+ + + +	+ + + +
	+ +	+ + + +	+ + + +	NA
	+ +	+ + + +	+ + + +	NA
CDDP Solution i.p.	+ + +	+ + +	+ + +	+ + +
	+ +	+ + +	+ + +	+ + +
	+ + +	+ + +	+ + +	+ + +
	+	+ + +	NA	+ + +

[a]Score of renal damage.
0, no significant lesion; +, modest renal damage involving <25% of the kidney; + +, mild, 25–50%; + + +, moderate, 50–70%, + + + +, severe, 75–100%; NA, no result due to animal death.

follow-up after treatments (Table 1). The greater the score, the severer the renal damage. There were 86.7% of the rats with moderate kidney damage (+ + +) in the solution i.p. group and 78.6% of the rats with moderate to severe kidneys damage (+ + + to + + + +). In the MS group, only one (6.25%) rat show moderate kidney damage.

Discussion

Cisplatin shows a broad spectrum of antitumor activity in animal tumor systems and in certain human cancers.[7] The chemosensitivity of human hepatocellular carcinoma tissues to cisplatin has been reported to be 51.5%.[8] Renal toxicity is the most serious dose-limiting factor in the clinical use of intravenous infusion of cisplatin.[7] Many attempts have been made to prevent or ameliorate platinum-induced nephrotoxicity such as slow infusion in conjunction with administration of saline and diuretic drugs, decreased dose for single courses of cisplatin, and wide spacing between individual doses of cisplatin kept within the margin of safety stated earlier.[9] In the case of saline and diuretic drug administration, the concentration of cisplatin in the tumor may be decreased because of drug excretion due to diuresis.

Because of its sustained release property, microencapsulation has been used to prevent or ameliorate platinum-induced nephrotoxicity. It has been demonstrated that using intraperitoneal administration in mice, the lethal toxicity of lactic acid oligomer microspheres was reduced to 57% of the soluble cisplatin.[10] Different types of cisplatin microspheres were investigated for transcatheter hepatic arterial chemoembolization (TAC).[11–14] Although TAC is recommended for the treatment of unresectable cases, this therapy can cause further damage to the liver. Because the anti-cancer activity of this drug depends not only on its concentration but also on the length of exposure, the cisplatin should be delivered to the target region at the desired concentration over a longer period of time.[10] Based on this theory, percutaneous intratumoral injection of cisplatin microspheres was evaluated. We selected poly(D,L-lactic acid) as a encapsulation material because it is a non-toxic, well-established, biocompatible and widely used biodegradable polymer,[15,16] which can be used as a novel drug delivery system (e.g. microspheres) for various clinical applications. The PLA-CDDP MS were injected intratumorally for several reasons. (1) The injected PLA-CDDP MS will stay interstitial of the tumor, cisplatin will slow release into interstitial fluid, and then interact with tumor cells or go to the blood stream via capillaries and the lymph system. Thus, 100% of the injected drug is initially in the tumor as a drug source; the drug may have a great opportunity to enter tumor cells before it goes to the blood. A high local concentration of cisplatin and a prolongation of the interaction between cisplatin and tumor cells will occur. The tumor cells will be exposed to non-protein bound cisplatin (the antineoplastic effect of cisplatin is related to the non-protein bound form). (2) By reducing the peak level of cisplatin in the blood, less systemic toxicity is anticipated and less hepatic damage compared to TAC because the treatment is confined to the tumor and does not occlude hepatic arteries. (3) Percutaneous injection is relatively simple, less invasive

and of low cost. (4) A higher dose of cisplatin (the total dose for several days) can be used in one single injection. The followed intravenous hydration and diuresis for reducing nephrotoxicity would not decrease the cisplatin concentration inside the tumor significantly. (5) Intratumoral injection can be used for palliative treatment of solid tumors located in other portions of the body, which are not suitable for surgery or TAC.

Intratumoral injection of cisplatin solution is not practical. Since cisplatin has poor solubility in water (1 mg/ml in 0.9 sodium chloride solution for i.v.), intratumoral injection of a cisplatin solution needs a large volume to accommodate the required dose. Escape of the solution to tissues surrounding the tumor is likely and the solution would be quickly washed out of the area by blood and lymph; a high blood cisplatin concentration will then occur, as seen in the CDDP i.t. group in our studies.

In our study, there was no significant difference between the MS group and solution groups (i.t. and i.p.) with regard to tumor 16 days post-injection. In an *in vitro* release study, PLA-CDDP MS produced sustained release in PBS at 37°C. *In vitro* and *in vivo* data from the present study suggest that cisplatin was released from the injected microspheres. The authors have described that because CDDP MS slowly released cisplatin into the peritoneal cavity, cisplatin levels in the circulating blood and extraperitoneal tissues remained very low.[10] Wakiyama *et al.*[18] reported that *in vitro* release of dibucaine-loaded PLA microspheres was faster than the corresponding *in vivo* release, because of a higher degradation rate *in vivo*. Encapsulated solid cisplatin was, in fact, in an aqueous environment (interstitial fluid) when the microspheres were injected intratumorally.

Only a very small amount of microspheres (2.7 mg/rat) was injected in our experiment, although the dosage of cisplatin (6 mg/kg) was high compared to clinical use. As described before, the microspheres remaining in the 'dead space' would cause an inaccuracy of cisplatin dosage and produces deviation in experiments. An optimal dose of PLA-CDDP MS to treat solid tumors would be desired in large animal experiments; because only a small proportion of microspheres remain in 'dead space' during larger dosage procedures, uncertainties in administered dose will therefore be negligible.

One of the characteristic features of acute cisplatin nephropathy is that tubular lesions are primarily localized in the corticomedullary regions, involving both the proximal and distal tubules. A single intraperitoneal dose of cisplatin (6 mg/kg body weight) has been shown to induce marked focal necrosis in the proximal and distal tubules with maximum lesions on day 7.[7] As shown in Figures 4 and 5, the mean BUN and creatinine of the rats that received cisplatin solution i.t. or i.p. were always higher than in the rats that received intratumoral CDDP MS. There was no advantage of i.t. injection of cisplatin solution to reduce nephrotoxicity compared with i.p. injection, because even more renal toxicity was induced by i.p. than i.p. administration. The most probable cause of animal death in the solution i.t. and i.p. groups was cisplatin-induced acute renal failure. The early fall in glomerular filtration rate in cisplatin-induced acute renal failure is in part due to reduced renal blood flow and lowered effective filtration pressure.[19] In comparison, early acute renal failure was not manifested in the MS group, as reflected by the stable serum BUN and creatinine levels. This reduced renal toxicity indicates a lower cisplatin concentration in the systemic blood circulation during the follow-up period since renal toxicity was reported to be dose related.[9] Some renal damage (+ + to + + +) was determined by histopathologic examination 16 days after treatment of CDDP MS i.t. The histopathologic results (Table 1) and kidney weight changes (Figure 6) reveal that the renal toxicity in the MS group has a tendency to increase lightly during 16 days, without significant change of serum BUN and creatinine levels. This may result from the accumulation of cisplatin in the kidneys or due to the fact that the doses of PLA-CDDP MS were not quite accurate in the small animal study. Perhaps the dosage may be also critical in intratumoral treatment using PLA-CDDP MS. Further investigation is needed. Nevertheless, one rat had only modest renal damage (+) by 16 days and the histopathologic examination revealed the residual tumor mass in this rat was mostly necrotic. These findings indicate that it is possible to produce tumor necrosis without significant renal alteration using PLA-CDDP MS.

Conclusion

Our experiment indicated that PLA-CDDP MS injected into solid tumors had antitumor potency and could diminish acute nephrotoxicity due to sustained release of cisplatin. Intratumoral injection of encapsulated cisplatin is a potential option for cancer chemotherapy.

Acknowledgments

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