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(Citation)
The Kobe journal of the medical sciences,45(5):229-243

(Issue Date)
1999-10

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/E0001037

Kobe J. Med. Sci. 45, 229/243, October 1999

EXPERIMENTAL STUDY ON PARATUMORAL INJECTION OF CISPLATIN-LOADED MICROSPHERES FOR GASTRIC CANCER

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INDEXING WORDS

drug-loaded microspheres; cisplatin; gastric cancer; paratumoral injection of anticancer drug; chemotherapy

SYNOPSIS

Microspheres from glycolide-L-lactide copolymers incorporating cisplatin (CDDP-MS) were prepared to evaluate the sustained release and anticancer effect by paratumoral injection on the gastric cancer with regional lymphnode metastases induced by VX2 tumor in rabbits. In the first set of experiment, the rabbits were divided into three groups subjected to treatment and compared the tissue cisplatin distribution. In the first group (CDDP-MS pt group) , 1 mg/kg of cisplatin was administered by the method of paratumoral injection in the form of CDDP-MS. In the second group (CDDP solution pt group) , the same dose was given in the form of CDDP aqueous solution in the same way and in the third group (CDDP solution iv group) , the same dose was intravenously administered. In the second set of experiment, after twice of each therapy the anticancer effects were compared between CDDP-MS pt and CDDP solution iv groups.In results, the platinum concentrations of the tumor and regional lymphnodes were 3.14 $\pm$ 6.22, 0.65 $\pm$ 0.79 μg/g in the first group, 0.43 $\pm$ 0.39, 0.16 $\pm$ 0.16 μg/g in the second group and 0.03 $\pm$ 0.01, 0.07 $\pm$ 0.05 μg/g in the third group, respectively.

Received for publication : April 20, 1999

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On the other hand, the tissue platinum concentrations in lung, liver and kidney in the CDDP-MS pt group were lower than those in the other two groups, resulting in reduction of the side effects. In the second set of experiments, the frequencies of liver and regional lymphnode metastases were 0/5, 0/5 in the CDDP-MS pt group, 1/5, 2/5 in the CDDP solution iv group and 3/3, 3/3 in control group respectively, with significant difference ($p<0.05$, CDDP-MS pt group vs. control group) and concerning the tumor diameters, there was significant difference between CDDP-MS pt group and control group ($p<0.01$) . These results suggested that the paratumoral injection of CDDP-MS had advantages of not only the good antitumor effect but also reduction of side effects.

INTRODUCTION

Accompanying surgical resection of the gastrointestinal tumor, removal of all of the metastatic lymphnodes is intended, however, all of minute metastatic lymphnodes cannot be identified and some may be left behind.[12] Although the adjuvant chemotherapy for the patients with lymphnode metastases has been performed, the prognosis has still been poor.[8,16]

In order to explore a more effective chemotherapy for the tumor and the regional lymphnode metastases, we have paid attention to the biodegradable particulate carrier system of CDDP-MS with lymphotropic targeting and controlled release.[9] In this experimental study, we have newly designed the paratumoral injection of CDDP-MS and compared the plasma and tissue distributions of drug and the anticancer effect with other two styles of treatment with cisplatin for gastric cancer model. As the result of study, we report on the selective delivery of high levels of cisplatin to primary tumor and regional lymphnodes, as well as reduced systemic toxicity and superior therapeutic efficacy of CDDP-MS on gastric cancer model.

MATERIALS AND METHODS

Drug preparation

The microspheres containing cisplatin were prepared with a solvent

evaporation method using oil-in-oil emulsion in the Institute for Frontier Medical Sciences, Kyoto University.[9,18] The weight-average molecular weight of glycolic acid/L-lactic acid copolymer (PGLA) was 14,000 and cisplatin (CDDP) was supplied from Nippon Kayaku Co., Ltd., Tokyo, Japan. PGLA was dissolved in 5 ml of dimethyl formamaide (DMF) containing 22 mg of CDDP. The PGLA/CDDP solution in DMF was added dropwise at 25 ℃ to 400g of liquid paraffin containing 10 wt% Span 80 under agitation. This emulsion was stirred by a disk-shaped stirring bar of 5 cm diameter at a constant speed of 400 rev./min., and then the temperature was gradually raised from 25 to 40 ℃ at a rate of 0.2 ℃/min., followed by maintaining at 40 ℃ for further 40 hr to evaporate DMF. The resulting microsphere were collected by centrifugation, washed four times with n-hexane and once with 2-propyl alcohol, and then dried under a reduced pressure until the residual DMF was virtually evaporated. The use of liquid paraffin as the dispersion phase was effective in reducing CDDP loss in the dispersed phase during microsphere preparation. A weighed amount of the CDDP-MS was dissolved in chloroform and then the CDDP was extracted into the water phase after addition of water into the chloroform solution. Drug loading of the CDDP-MS was 5.0 %. The CDDP concentration of the sample was determined by measuring the absorbance at 265.95 nm on a flameless atomic absorption spectrometer (atomic absorption spectroscope type Z-7000, Hitachi Co. Ltd., Tokyo, Japan).[15]

The figure of the CDDP-MS was spherical and the surface was smooth without porous and crack, and the size was ranged from 5 to 40 μm. After coating with platinum, the surface of microspheres was observed by scanning electron microscopy (SEM) with Hitachi Model S-450 manufactured by Hitachi Co. Ltd., Tokyo, Japan (Fig. 1) . SEM obsevation of the microspheres was performed after drug release and subsequent washing in 2-propanol and vacuum-drying. To observe the microsphere cross-section by SEM, the microsphere was placed in aqueous polyvinyl alcohol solution and cut with a microtome after cooling to -24 ℃ with a cryostat. About release rate of the CDDP-MS in vitro, fifty percent of the cisplatin was available for relatively fast release during 24 hours (burst effect), and another 25 % was then released at a slower rate over 21 days .[18]

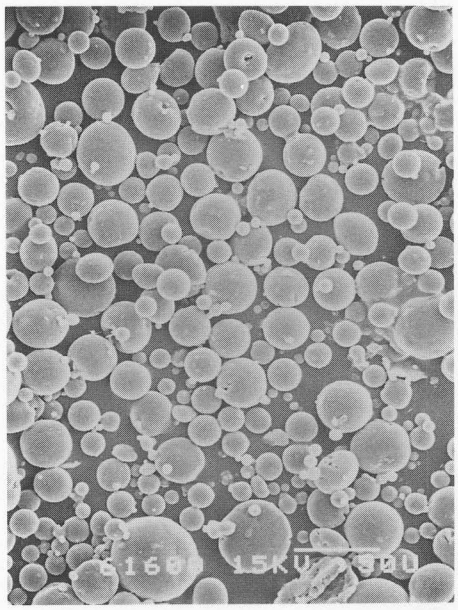

Fig. 1. A scanning electron microscopic view of CDDP-MS.

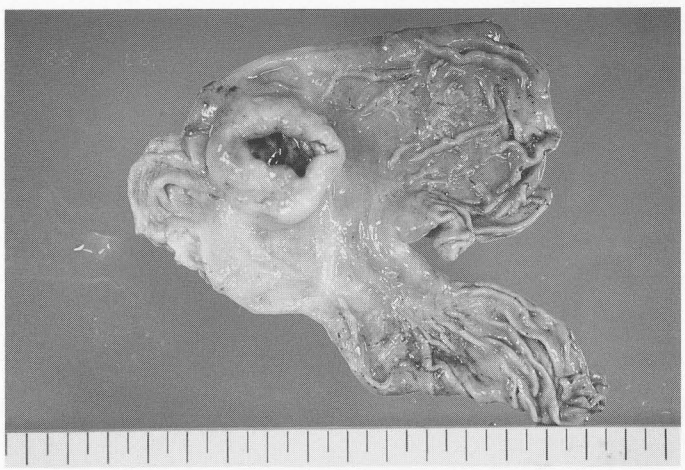

Fig. 2. VX2 gastric cancer model.

Animal model

A total of 42 Japanease white rabbits (female, mean body weight of 2.0 kg) from the same large colony were used in the first and second set of experiments. The rabbits were maintained under standard conditions and fed on standard rabbit chow and tap water freely. The use of animals in this study confirmed to the guidelines of the NIH and approved by our institutional Animal Care Committee. Gastric cancer model was produced by VX2 carcinoma cells[17] (2×10^6 cells/0.2 ml phosphate-buffered saline, in the same manner established by Nakamura *et al.*[14]) implanted with a 26-gauge needle into the anterior wall of the middle third of stomach, during midline laparotomy under the intravenous anesthesia by pentobarbital sodium (30 mg/kg). VX2 carcinoma cells maintained in the femoral muscle of rabbits, from generation to generation, were provided by the Kyowa Hakko Co., Ltd., Osaka, Japan. Three weeks after tumor inoculation, the model tumor grew up into the protruding type cancer with ulceration, accompanying the regional lymphnode metastases which are around the truncus of left gastric artery and liver metastases (Fig. 2).

Drug administrations

Three weeks after tumor inoculation, the rabbits were divided randomly into the three groups and administered in 1.0 mg/kg body weight of cisplatin. In the first group, CDDP-MS was administered by the method of paratumoral injection under laparotomy (CDDP-MS pt group). And in the second group, CDDP solution was given in the same way (CDDP solution pt group) and in the third group, CDDP solution intravenously administered (CDDP solution iv group). Paratumoral injection of drug was performed as injection into the bilateral side of tumor through the serosal side with a 20-gauge needle under midline laparotomy. The paratumoral injection of CDDP aqueous solution and intravenous administration via ear vein were used by aqueous solutions of cisplatin for injection (Landa inj, Nippon Kayaku Co., Ltd., Tokyo, Japan). Plasma platinum concentrations of blood samples were measured at 1, 3, 7 and 14 day after drug administration, and two weeks after each therapy, the rabbits were sacrificed and a half of tumor, regional lymphnodes around the truncus of left gastric artery, right lung, left lobe of liver and right kidney were dissected for tissue platinum

analysis. One hour before sacrifice, BrdU 10 mg/kg was administered intravenously and BrdU uptake rates of the advancing margin of tumor tissue were determined by the count of percentage of 1,000 cells of tumor. As an analogue of thymidine, 5-bromo-2'-deoxyuridine (BrdU) is taken into the S-phase cells of cell cycle, therefore, BrdU labeling index is used as an index of proliferating activity of tumor by the method of immunohistochemical technique using monoclonal antibody.[2,4] And the tumor diameter was measured as an anticancer effect.

In the second set of experiment, concerning about the anticancer effects and side effects, the rabbits were assigned to three groups and treated twice at an interval of one week, by paratumoral injection of CDDP-MS or by intravenous administration of same dosage in the first set of experiment. The first administration of each drug was performed two weeks after tumor inoculation and the second, two weeks after the first, and two weeks after the second, we sacrificed the rabbits to investigate the frequency of metastasis in liver and regional lymphnode and the tumor diameter, comparing with the control group which didn't perform any treatment. Concerning the tissue distribution of cisplatin, a half of tumor and right kidney were taken for measurement of platinum concentration. And we sampled the blood serum and measured serum levels of blood urea nitrogen (BUN) , serum creatinine (Cr) , serum glutamic oxaloacetic transaminase (GOT) and lactate dehydrogenase (LDH) with measurement of body weight change, reflecting the side effect of drug.

Statistical analysis of data

All differences were analyzed with a two-tailed Student's t test or *Fisher's exact probability* test. P values less than 0.05 were considered significant. All data are expressed and displayed as mean $\pm$ standard deviation (SD) unless stated otherwise.

RESULTS

Changes with time in platinum concentrations of blood plasma were compared among the three groups. In the CDDP-MS pt group, the platinum

rose slowly from the first to the third day and persisted at high level because of sustained release of CDDP-MS, while in the other two groups, they decreased linearly over time (Fig. 3) . The platinum concentrations of the tumor and regional lymphnodes were respectively, 3.14 ± 6.22, 0.65 ± 0.79 μg/g in the CDDP-MS pt group, 0.43 ± 0.39, 0.16 ± 0.16 μg/g in the CDDP solution pt group and 0.03 ± 0.01, 0.07 ± 0.05 μg/g in the CDDP solution iv group. On the other hand, the tissue platinum concentrations in lung, liver and kidney in the CDDP-MS pt group were lower than those in the other two groups, resulting in reduction of the side effects (Table I) . This result showed that the levels of platinum in the tumor and the regional lymphnodes were maintained higher for 14 days in the CDDP-MS pt group than those in the CDDP solution pt or CDDP solution iv groups. On the other hand, tumor diameters in each therapy showed no significant differece among the three groups, but the BrdU labeling indexes were respectively 12.3 ± 2.8 %, 16.5 ± 10.8 % , 23.1 ± 9.7 % (p<0.01, CDDP-MS pt group vs. CDDP solution iv group) (Table II) .

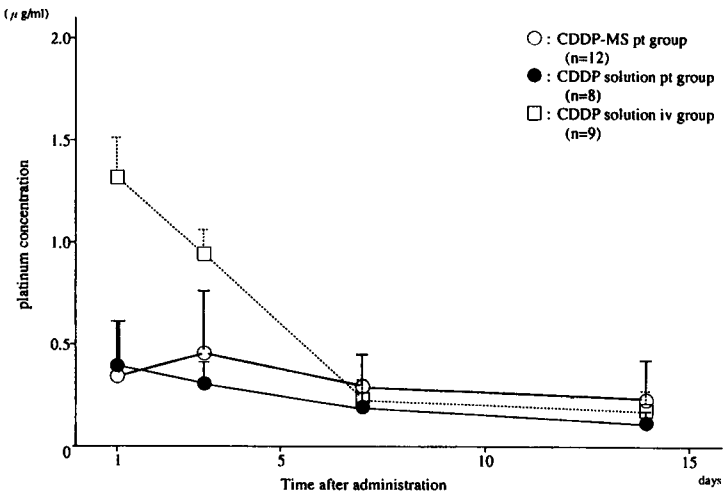

Fig. 3. Serum concentrations of platinum.

In the second set of experiments, the frequencies of metastases in liver and regional lymphnodes were respectively, 0/5, 0/5 in the CDDP-MS pt group, 1/5, 2/5 in the CDDP solution iv group and 3/3, 3/3 in control group with significant difference ($p<0.05$, CDDP-MS pt group vs. control group) . The tumor diameters were respectively 18.4 $\pm$ 7.4 , 35.6 $\pm$ 18.4 and 41.3 $\pm$ 6.5 mm with significant difference ($p<0.01$, CDDP-MS pt group vs. control group) (TableⅢ) . Concerning the platinum concentrations of the tumor and kidney between the CDDP-MS pt group and the CDDP solution iv group, significant difference was resulted in those of kidney ($p<0.01$) (TableⅣ) . The laboratory data of serum samples, BUN, Cr, GOT, LDH and each change of body weight showed no differeces among the three groups (TableⅤ) .

DISCUSSION

Surgical lymphadenectomy with resection for gastrointestinal cancer should be expected for en bloc resection of the regional lymphnodes, however, all of the micrometastatic lymphnodes cannot be identified and some may be left behind, because of anatomical restriction and complex network of lymphatics.[19] The one of the reasons for poor postoperative prognosis is the high incidence of lymphnode metastasis especially in esophageal and gastric cancer.[10,13] In the conventional chemotherapy, namely intravenous administration, there may be high blood concentration of anticancer drug, sequentially to high possibility of side effects. Therefore, as a supportive chemotherapy with the reduction of systemic side effects, we have selected the regional chemotherapy of CDDP-MS and designed the paratumoral injection of the drug which can be repeatedly performed by endoscopy.

Slow-release devices, like as microspheres, emulsions and liposomes *et al.*, were chiefly reported in regard to intraperitoneal administration for carcinomatosa peritonitis model or intraperitoneally inoculated tumor model.[6,11,20] Clinically, Hagiwara *et al.*[7], as a pilot study, used the microsphere loading anticancer drug for the treatment of malignant ascites induced by cancers of digestive organs and showed a marked therapeutic benefit with small systemic side effects. Concerning intratumoral or local injection of slow-release drugs, Deurloo *et al.*[1]

Table Ⅰ. Tissue platinum concentration of tumor, regional lymphnode, lung, liver and kidney.

Experimental group	tumor	lymphnode	lung	liver	kidney
CDDP-MS pt group (n=12)	3.14 ± 6.22	0.65 ± 0.79	0.05 ± 0.03	0.17 ± 0.23	0.07 ± 0.04
CDDP solution pt group (n=8)	0.43 ± 0.39	0.16 ± 0.16	0.06 ± 0.02	0.16 ± 0.16	0.25 ± 0.26
CDDP solution iv group (n=9)	0.03 ± 0.01	0.07 ± 0.05	0.15 ± 0.08	0.42 ± 0.2	0.47 ± 0.32

* : $p < 0.05$
** : $p < 0.01$
*** : $p < 0.001$

(mean ± SD μ g/g)

Table Ⅱ. Tumor diameter and BrdU labeling index.

Experimental group	tumor diameter (mm)	BrdU labeling index (%)
CDDP-MS pt group (n=12)	22.7 ± 11.8	12.3 ± 2.8
CDDP solution pt group (n=8)	27.4 ± 22.4	16.5 ± 10.8
CDDP solution iv group (n=9)	25.5 ± 18.8	23.1 ± 9.7

* : $p < 0.01$

(mean ± SD)

Table Ⅲ. Frequency of liver and lymphnode metastases and tumor diameter (in the 2nd set of experiment).

Experimental group	metastasis		tumor diameter # (pre-therapy)
	liver	lymphnode	
CDDP-MS pt group (n=5)	0/5	0/5	18.4 ± 7.4 (7.8 ± 1.8)
CDDP solution iv group (n=5)	1/5	2/5	35.6 ± 18.1 (9.0 ± 3.7)
Control group (n=3)	3/3	3/3	41.3 ± 6.5

* : $p < 0.05$
** : $p < 0.01$

: mean ± SD mm

Table Ⅳ. Tissue platinum concentration of tumor and kidney (in the 2nd set of experiment).

Experimental group	platinum concentration	
	tumor	kidney
CDDP-MS pt group (n=5)	8.94 ± 11.2	0.096 ± 0.060 *
CDDP solution iv group (n=5)	0.09 ± 0.04	1.022 ± 0.517 *
	*: $p < 0.01$	mean ± SD μ g/g

Table Ⅴ. Serum BUN, creatinine, LDH and change of body weight (in the 2nd set of experiment).

Experimental group	BUN (mg/dl)	Cr (mg/dl)	GOT (IU/l)	LDH (IU/l)	change of weight# (%)
CDDP-MS pt group (n=5)	19 ± 5.6	0.7 ± 0.1	35 ± 31.2	457 ± 112	103.1 ± 7.0
CDDP solution iv group (n=5)	18.6 ± 3.9	0.9 ± 0.2	26.6 ± 24.5	525 ± 100	101.4 ± 8.8
Control group (n=3)	15.1 ± 3.2	1.0 ± 0.2	32 ± 10.8	569 ± 98	102.3 ± 4.3

: weight at autopsy/weight at first therapy

demonstrated that intratumoral administration of solid slow-release rods prepared either from starch or from three different polyether-hydrogel formulation could result in high tumor responses and Hagiwara *et al.*[5] reported the effectiveness of a new dosage formulation consisting of an anticancer drug bounded to activated carbon particles. Recently, the CDDP-MS was also reported to be molecularly homogenous distribution of cisplatin molecules throughout the matrix of PGLA and obtained the long-term sustained release.[9]

We planed an effective chemotherapy for the lymphnode micrometastasis via lymphatics from gastric primary tumor with reduction of systemic side effects of anticancer agents. For this purpose, we selected PGLA microspheres as a biodegradable lymphotropic carrier and cisplatin as a more effective and widespreading anticancer drug for gastric cancer. The antitumor activity of cisplatin depends on both its concentration and its exposed time,[3] therefore it is more effective in the formulation of controlled release device than the CDDP aqueous solution. In this time, CDDP-MS was designed to release 70% of the incorporated cisplatin slowly during a period of 3 weeks at the site where the particles of CDDP-MS were retained.[9] We expected that the CDDP-MS of which the size was ranged from 5 to 40 μ m had a characteristic of selectively lymphotrophic delivery to the regional lymphnodes. Because it is supposed that large particles are large enough to pass through lymph-capillary wall and not to pass through blood-capillary wall, and on the other hand, small particle as cisplatin pass easily through the both with high plasma and lymph cisplatin concentration. These characteristics suggest that CDDP-MS is retained around the tumor releasing CDDP slowly with high local tissue concentration and the plasma cisplatin concentration was maintained at low level. And the tissue cisplatin concentrations of kidney, lung and liver were lower due to low plasma cisplatin concentration. But between CDDP-MS pt and CDDP solution iv groups in both experiments, platinum concentrations of the tumor were not significantly different because of the wide range in the former and about tumor diameters there was not significant difference in spite of the significant difference of BrdU labeling index. We guessed two possibilities related to those results. The one is that the platinum concentration might not be high enough to suppress the growth of tumor for relatively advanced tumor. The other is that the interval of tumor inoculation might be long. In the second set of experiment, in the CDDP-MS pt group, platinum concentration of tumor showed very high level and anticancer effect was good without lymphnode and liver metastases. The results indicate that the frequency of treatment is related to anticancer effect and in comparison with kidney platinum concentration of the CDDP solution iv group, there is a possibility to administrate more dose in the CDDP-MS pt group. And in order to get better experimental results, we need to adjust dose, frequency and interval of

the CDDP-MS pt injection.

Concerning the administration method of CDDP-MS, the paratumoral injection was endoscopically repeatable and technically easy. Morever this therapy was applicable for inoperative cases by reason of physically untolerance of surgery or unresectable cancer cases. And we expect that this therapy is very suitable as a preoperative chemotherapy for gastrointestinal cancers with suspicious lymphnodes metastases, into which primary lesions we can inject the drug endoscopically.

In conclusion, the paratumoral injection of CDDP-MS yielded much higher platinum concentrations of the tumor and the regional lymphnodes than did injection of free CDDP solution or intravenous administration. On the other hand, it reduced the platinum concentration in the other organs and resulted in a reduction of systemic toxicity. Therefore, we conclude that PGLA CDDP-MS may represent a useful and effective means of chemotherapy for gastric cancer with metastatic lymphnodes.

ACKNOWLEDGEMENTS

The authers wish to thank Dr. Hyon, S. H., Institute for Frontier Medical Sciences, Kyoto University, for preparation and provision of cisplatin-loaded PGLA microspheres.

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