

## A Study of Embolizing Materials for Chemo-embolization Therapy of Hepatocellular Carcinoma: Antitumor Effect of *cis*-Diamminedichloroplatinum(II) Albumin Microspheres, Containing Chitin and Treated with Chitosan on Rabbits with VX<sub>2</sub> Hepatic Tumors

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As an effective therapy for hepatocellular carcinoma, hepatic arterial chemo-embolization therapy has been widely used, and many embolizing materials have been extensively investigated. In the present study, we prepared various types of *cis*-diamminedichloroplatinum(II) (CDDP) albumin microspheres using chitin and chitosan, both of which have attracted considerable attention as new non-toxic biological polymer materials having favorable characteristics such as immune adjuvant activity, biological compatibility, and biodegradation.

Hepatic artery of rabbit hepatic cancer models, which had transplanted VX<sub>2</sub> tumors, were embolized with various types of microspheres. The anti-tumor effects and tumor-targeting of the microspheres, and the effects of the microspheres administration on the hepatic tissue were investigated.

As a result, anti-tumor activity of the microspheres was increased by the addition of chitin-containing or chitosan treated materials; tumor growth rates of chitin addition and chitosan treated groups were approximately 160% and 120%, respectively, and were significantly lower than that of the non-treatment groups with a rate of approximately 580%. However, complete inhibition of tumor growth might have been impossible. Anti-tumor activity was increased by the addition of chitin-containing or chitosan treated materials. Whereas the growth inhibitory effect was insufficient, in order to potentiate anti-tumor activity, higher CDDP contents and sustained release of CDDP at a high level from microsphere and so on should be essentially improved for the near future. The CDDP level in hepatic tissue following the administration of microspheres was increased by adding chitin to the microspheres or by treating the microspheres with chitosan. However, no difference was observed in the platinum concentration between the tumor portion and normal portion; thus, microspheres showed no tumor-targeting. Also, no effects of microsphere administration on functions of the liver or kidneys were observed. Based on these observations, we concluded that chitin or chitosan had an antitumor effect in VX<sub>2</sub> rabbit hepatic tumor models and thereby were effective as embolizing materials.

**Keywords** chitin; chitosan; *cis*-diamminedichloroplatinum(II); albumin microsphere; anticancer activity; hepatocellular carcinoma

As therapies for hepatocellular carcinoma, surgical procedure, hepatic artery embolization and ethanol local injection are presently available. However, as single therapies, these conventional approaches have limitations: Recurrence and polycentric generation after oncotomy often occur. Therefore, in order to improve the efficacy of these single therapies, several studies on combined therapy, which employs chemotherapy or immunotherapy, have been performed.<sup>1-4</sup> In our previous studies,<sup>5,6</sup> we performed several *in vitro* experiments as well as experiments in healthy mongrel dogs and reported the usefulness of *cis*-diamminedichloroplatinum(II) (CDDP) albumin microspheres containing chitin or chitosan as embolizing materials. In the present study, as a first step in clinical application, we prepared rabbit hepatic cancer models by transplanting VX<sub>2</sub> tumors into rabbits, administered them with CDDP albumin microspheres treated with chitin or chitosan *via* the hepatic artery, and investigated the anti-tumor effect of the therapy, time-course change of blood CDDP level, CDDP level in hepatic tissue, and the embolization effects and tumor-targeting of the microspheres. The obtained results are reported below.

### Experimental

**Reagents** CDDP powder was kindly supplied by Nippon Kayaku Co., human serum albumin by the Green Cross Co., chitin and chitosan (70% of deacetylation) by Nakarai Tesque Co., Ltd., sodium pentobarbital (Nenbutal) injection by Dainippon Pharmaceutical Co., Ltd., All other

reagents employed were commercial special-grade products.

**Preparation of CDDP Albumin Microspheres, Chitin-Containing CDDP Albumin Microspheres, and Chitosan Treated Chitin-Containing CDDP Albumin Microspheres** Albumin microspheres, chitin-containing albumin microspheres were prepared according to the methods described in a previous paper<sup>4</sup>: CDDP powder and chitin (1.5% concentration) were mixed to a fine powder with a mortar and pestle. 25% of albumin solution was added and mixed well. This solution was added to toluene-chloroform mixed with ethylcellulose and emulsified according to the method of preparation for w/o emulsion, and hardened with glutaraldehyde. The products were washed with acetone, air-dried at 50 °C for 2 h. To obtain chitosan treated chitin-containing CDDP albumin microspheres, chitin-containing CDDP albumin microspheres were stirred for a set time in an acetic acid solution of chitosan (1.5%) and washed, and then air-dried at 50 °C for 2 h. These microsphere were sieved into grades (20—36 μm), and sterilized by dry heat at 135 °C for 2 h, and used as samples.

**In Vitro Release Test** The release test was performed according to the method described in a previous paper.<sup>4</sup> As the releasing solution, physiologic saline was used. As for the device, a releasing test device made by Toyama Sangyou Co. was used. Measurement was performed at 37 °C.

**Preparation of VX<sub>2</sub> Rabbit Hepatic Tumor Model** Under anesthesia using pentobarbital sodium the abdomen of Japanese male albino rabbits (body weight 2.8—3.5 kg) was opened and VX<sub>2</sub> tumor 2 × 2 × 2 mm in size was transplanted under the capsular of the left lobe of the liver. Ten days after the transplantation, the abdominal of the animals were again opened under anesthesia, and the longitudinal and latitudinal diameters of the tumors were measured. Then the following experimental procedure was performed.

Ten days after the tumor transplantation, the abdominal of the animals were opened and test agents (one mg of CDDP per kg body weight) were administered *via* the left hepatic artery using a wing type needle (30 gauge). Animals were killed 7 d after administration, and longitudinal and latitudinal diameters were measured. As control groups, a non-treated

animal group and an albumin microspheres administered animal group were prepared. After the administration, blood samples were also collected at a certain time interval from the ear vein and the blood CDDP level of each sample was determined.

**Anti-tumor Effect** In order to monitor tumor growth, longitudinal and latitudinal diameters of the tumors were measured before and after microsphere administration. Data on longitudinal and latitudinal diameters were then employed as the index for anti-tumor effect. An anti-tumor effect was also evaluated by the gross and histopathological observation of the liver.

The tumor growth rate was calculated according to the following formula:

$$\text{tumor growth rate (\%)} = \frac{\text{product of longitudinal and latitudinal diameter 17 d after transplantation} - \text{product of longitudinal and latitudinal diameter 10 d after transplantation}}{\text{product of longitudinal and latitudinal diameter 10 d after transplantation}} \times 100$$

**Measurement of CDDP Level in the Hepatic Tissue** Rabbits were killed 7 d after administration of microspheres and the livers were extirpated. Platinic content in the tumor portion of the dissected livers, normal portion adjacent to the tumor, and normal portion distal to the tumor was determined according to the method described in a previous paper<sup>5)</sup>: Concentrated nitric acid was added to a liver specimen and it was heat-decomposed. The product was then neutralized by adding sodium hydroxide and sodium carbonate. Then, platinum was chelated and extracted using chloroform.

**Results**

**CDDP Release from Each Type of Microsphere *in Vitro***

Figure 1 shows the CDDP releasing rate of each type of microsphere. The CDDP releasing rate of CDDP albumin microspheres, chitin-containing CDDP albumin microspheres and chitosan treated chitin-containing CDDP albumin microspheres were significantly different from each other: The time required for 50% release was about 0.5 h in CDDP albumin microspheres, 4.5 h in chitin-containing CDDP albumin microspheres, and 10.5 h in chitosan treated chitin-containing CDDP albumin microspheres.

**CDDP Release in the Rabbit VX<sub>2</sub> Hepatic Carcinoma Model**

Figure 2 shows the change of the blood concentration of CDDP (platinum). CDDP albumin microsphere showed at approximately 0.6 μg/ml 1 h after administration, then gradually decreased and was below the limit of measurement within 6 d. Chitin-containing CDDP albumin microsphere showed at approximately 0.7 μg Pt/ml 1 h after administration, then gradually decreased and was still 0.2 μg Pt/ml 7 d after. Chitosan treated chitin-containing CDDP albumin microsphere showed at approximately 0.8 μg Pt/ml 1 h after administration. CDDP release was

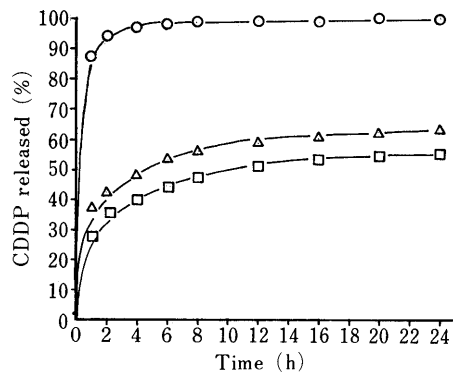


Fig. 1. Release Profiles of CDDP from CDDP Microspheres

Each point represents the mean ± S.D. of five experiments. Determination of CDDP was performed using an atomic absorption spectrophotometer. ○, CDDP albumin microsphere; △, chitin-containing CDDP albumin microsphere; □, chitosan treated chitin-containing CDDP albumin microsphere.

sustained and the blood platinic level was still approximately 0.3 μg Pt/ml 7 d after administration.

**Platinic Content in Hepatic Tissue** Table I lists platinic content in hepatic tissue 7 d after administration of microspheres. Platinic content in hepatic tissue differed among CDDP albumin microspheres, chitin-containing CDDP albumin microspheres, and chitin-containing chitosan treated CDDP albumin microspheres. On the contrary, in each microsphere administration, the platinic content was not significantly different between the tumor portion and the normal portion.

**Anti-tumor Effect** Table II shows the tumor growth rate in the rabbit hepatic carcinoma models. Tumors grew significantly in the non-treatment group, showing a growth rate of 577.7 ± 67.3%. The albumin microsphere administration group showed a growth rate of 404.5 ± 23.5%,

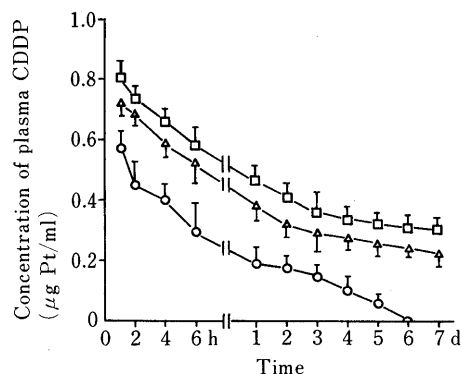


Fig. 2. Time Course of Plasma CDDP after Arterial Injection of CDDP Microsphere

Each point represents the mean ± S.D. of three rabbits. Determination of CDDP was performed using an atomic absorption spectrophotometer. ○, CDDP albumin microsphere; △, chitin-containing CDDP albumin microsphere; □, chitosan treated chitin-containing CDDP albumin microsphere.

TABLE I. The Assay of CDDP in Rabbit Liver

Region	CDDP concentration (Pt μg/g)		
	A	B	C
Tumor	1.09 ± 0.20	2.04 ± 0.11	2.19 ± 0.22
Normal portion adjacent to tumor	0.82 ± 0.06	1.80 ± 0.18	1.95 ± 0.09
Normal portion distant from tumor	0.85 ± 0.08	1.46 ± 0.11	1.66 ± 0.19

The data represent the mean ± S.D. of three rabbits. CDDP was measured by atomic absorption spectrophotometer. A was CDDP albumin microsphere, B was chitin-containing CDDP albumin microsphere, and C was chitosan treated chitin-containing CDDP albumin microsphere.

TABLE II. The Mean Growth Rate of VX<sub>2</sub> Tumor in the Liver of Rabbits

Microsphere	Growth rate% (mean ± S.D.)
Non-administration	577.7 ± 67.3
Albumin microsphere	405.5 ± 23.5
CDDP albumin microsphere	275.5 ± 32.7
Chitin-containing CDDP albumin microsphere	157.6 ± 19.4
Chitosan treated chitin-containing CDDP albumin microsphere	118.9 ± 19.5

The data represent the mean ± S.D. of three rabbits.

indicating trends of tumor growth suppression, however, the difference was not significant ( $p < 0.05$ ). The CDDP albumin microsphere administration group showed a growth rate of  $275.5 \pm 32.7\%$ , indicating suppression of tumor growth. Chitin-containing and chitosan treated chitin-containing CDDP albumin microsphere groups showed tumor growth rates of  $157.6 \pm 19.4\%$  and  $118.0 \pm 19.5\%$ , respectively, indicating significant tumor suppression.

### Discussion

Various types of CDDP albumin microspheres were prepared using chitin and chitosan, and for each microsphere group, the time course change of blood CDDP level, CDDP level in liver tissue, embolizing effect, anti-tumor effect, and tumor-targeting were examined. As a result, by adding chitin to CDDP albumin microspheres or treating them with chitosan, the release of platinum into the blood became slow and sustained. Also, the platinum level in liver tissue tended to increase. These observations were explained as follows: Addition of chitin improved the CDDP retention by microspheres and treatment with chitosan inhibited degradation of microspheres caused by biological enzymes. There was a significant difference in the platinum level in liver tissue among each type of microsphere, but there was no significant difference in the level between the tumor portion and non-tumorous portion in each group. Based on this observation, microspheres were concluded to have no tumor-targeting. The anti-tumor effect was enhanced by adding chitin to CDDP albumin microspheres or by treating it with chitosan in accordance with the increased CDDP levels in the liver and plasma. In the histopathological study, non-treatment group, tissue inside the tumor had formed macular lysed necrosis and viable tumor cells were extensively scattered around the necrosis. In the CDDP albumin microsphere administration group, viable tumor cells were observed. No significant difference was observed between the non-treatment and CDDP

albumin microsphere administration groups. In both the chitin-containing and chitosan treated chitin-containing groups, the tumor had necrotized but was not a massive type, and viable tumor cells were observed. Also, in animals that received microsphere administration including albumin microsphere, a certain degree of necrosis of normal liver tissue, which was caused by embolization, was observed. The reasons for these findings are believed to be as follows: Because the CDDP contents in microspheres employed in the present study were low, larger doses of administration were required. As a result, complete embolization of the hepatic artery adjacent to the tumor failed, and in some cases, only the hepatic artery distal to the tumor might be embolized. VX<sub>2</sub> tumors, having a shorter doubling time, grow too fast, and satisfactory anti-tumor effects might not be achieved. As for blood biochemical, a transient increase in the glutamic oxalacetic transaminase (GOT), total bilirubin, levels was observed, however, they returned to the normal range on the following day. All other parameters were within the normal range. As for future subjects, higher CDDP contents in microsphere, and the sustained release of CDDP at a high level from microsphere should be further studied.

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