A Study of Embolizing Materials for Chemo-embolization Therapy of Hepatocellular Carcinoma: Effects of Particle Size and Dose on Chitin-Containing cis-Diamminedichloroplatinum(II) Albumin Microsphere Antitumor Activity in VX₂ Hepatic Tumor Model Rabbits

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We prepared chitin-containing cis-diamminedichloroplatinum(II) (CDDP) albumin microspheres with various particle sizes, and investigated in vitro CDDP release; the antitumor effect towards VX₂ tumor introduced into rabbits was then examined. It was found that the rate of release of CDDP from chitin-containing CDDP albumin microspheres in vitro was increased with reduced particle size.

Administration of microspheres to VX₂ tumor-bearing rabbits via the hepatic artery resulted in different profiles of plasma platinum concentration depending on the particle size, and a higher concentration of platinum was released from the beginning of administration as particle size was reduced.

The platinum content in hepatic tissue following the administration of CDDP microspheres was increased as the particle size decreased, although the rate of increase was not uniform. The antitumor effect of CDDP assessed by the suppression of tumor growth tended to be higher when microspheres of smaller sizes were used. However, no significant difference was observed in tumor growth rate between rabbits injected with microspheres smaller than 20 μm and those injected with sizes between 20 and 37 μm (p > 0.05).

We also examined the relationship between the CDDP dose and antitumor effect using microspheres of less than 20 μm and observed a dose-dependent antitumor effect. No significant difference was observed, however, between 2 and 4 mg eq CDDP/kg dose levels (p > 0.05). From these results, we concluded that microsphere size and CDDP dose were strongly correlated with the augmentation of antitumor effect of chitin-containing CDDP albumin microspheres used in chemo-embolization therapy via the hepatic artery.

Keywords chitin; particle size; antitumor effect; chemo-embolization; cisplatin microsphere; administration dose

Hepatic arterial chemo-embolization therapy in the hepatic artery has been tested for the treatment of hepatocellular carcinoma, and several clinical investigations have been reported.¹ —¹⁰ Attractive lymphokine activated killer (LAK) therapy has drawn attention and obtained good results,¹¹,¹² and among the biodegradable biopolymer materials tested is chitin, which is known to have immunopotentiating and immunoregulatory qualities. In previous reports,¹³ —¹⁵ we prepared chitin-containing cis-diamminedichloroplatinum(II) (CDDP) albumin microspheres for application in chemo-embolization therapy in the hepatic artery. Then, we examined CDDP content and physical properties of the microspheres or antitumor activity against hepatic VX₂ tumor introduced into model rabbits on chitin concentration.

CDDP content in the microspheres and the specific surface area were greatly affected by the addition of chitin, and these indexes were increased with the increase of chitin concentration. Antitumor effect was also increased in proportion to the amount of added chitin indicating the usefulness of this substance augmentation of antitumor activity of CDDP. However, this therapy did not suppress tumor growth completely, and some problems on the embolic state and CDDP dose were left unresolved.

In this study, chitin-containing CDDP albumin microspheres with various particle sizes were prepared, based upon the assumption that the factors affecting embolic state were particle size and CDDP dose. We examined the relationship between drug release and antitumor effect on particle size of these microspheres and dose of CDDP.

MATERIALS AND METHODS

Reagents CDDP powder was generously supplied by Nippon Kayaku Co. Human serum albumin by the Green Cross Co., and chitin were obtained from Nacalai Tesque Co., Ltd., and CDDP solution by Nippon Kayaku Co. All other reagents employed were commercial special-grade products.

Preparation of Chitin-Containing CDDP Albumin Microspheres These microspheres were prepared as previously described.¹³ CDDP powder and chitin (concentration: 6.0%) were finely powdered, and an albumin solution was added. This suspension was added to toluene-chloroform mixed with ethyli cellulose and emulsified according to the method of preparation for W/O (water-in-oil) emulsion, and hardened with glutaraldehyde. The products were washed with acetone, air-dried at 50°C for 2 h, and sieved through different meshes to separate the sizes: A (less than 20 μm ), B ( 20—37 μm ) and C ( 37—74 μm ), followed by dry heating
sterilization at 135 °C for 2 h, and used as samples. 

**In Vitro Release Test** The release test was performed according to the method described. Using physiologic saline as the releasing solution and a releasing test device made by Toyama Sangyou Co., measurements were performed at 37 °C. The CDDP release from the microspheres into solution was measured by atomic absorption spectrophotometry (Hitachi, Z-9000).

**Preparation of VX₂, Rabbit Hepatic Tumor Model** VX₂ tumor was obtained from the Funabashi Farm, and was established as a tumor cell line in our laboratories. Japanese male albino rabbits weighing 2.5 to 3.0 kg were used throughout the experiments. VX₂ tumor was transplanted under the capsular parenchyma of the left lobe of the liver as described. During a laparotomy 10 d after the transplant each VX₂ tumor mass was measured (longitudinal and latitudinal diameters). Next, using a winged needle (30 gauge), chitin-containing CDDP albumin microspheres and CDDP solution were injected via the left hepatic artery. Animals were killed 7 d after administration, and the longitudinal and latitudinal diameters of the tumors were again measured. The control group, was a chitin-containing albumin microsphere administered animal group. Tumor size in the rabbits used in this study ranged 112.5 ± 26.7 mm². After administration, blood samples were also collected at certain time intervals from the ear vein and the plasma platinum concentration of each sample was determined by atomic absorption spectrophotometry. The area under the plasma platinum concentration–time curve (AUC) was calculated by a trapezoidal method.

**Antitumor Effect** Using the product of the tumors longitudinal and latitudinal diameters obtained after administration of chitin-containing CDDP albumin microspheres and CDDP solution as an index, the following equation was applied to assess the antitumor effect. The tumor growth rate was calculated according to the following formula:

\[
P_{17} - P_{10} = \frac{(P_{17} - P_{10})}{P_{10}} \times 100
\]

\( P_{17} \) product of longitudinal and latitudinal diameter 17 d after transplantation; \( P_{10} \) product of longitudinal and latitudinal diameter 10 d after transplantation.

**Measurement of Platinum Content in the Hepatic Tissue** Hepatic tissue platinum content was determined as described. The rabbits were killed 7 d after administration of CDDP microspheres and CDDP solution, and the livers were extirpated. The extracted liver and concentrated nitric acid was thermolyzed at 80 °C for 5 h. After air cooling of each sample, sodium hydroxide and sodium carbonate was added. Then, sodium diethyl dithiocarbamic acid, a platinum chelating agent, was added and the mixture was heated and cooled, followed by extraction with chloroform. The chloroform layer was evaporated to dryness using an evaporator, and the residue was dissolved in methanol to yield a sample. The platinum level was measured by atomic absorption spectrophotometry.

**RESULTS**

**CDDP Release in Vitro** Figure 1 shows that CDDP released from different size chitin-containing CDDP albumin microspheres increased with reduction in particle size. The total amount of CDDP released after 24 h was 47.5, 26.3 and 18.1% for microsphere A, B and C, respectively.

**Plasma Platinum Concentration** Figure 2 shows the plasma concentration time course of platinum after administration of different size chitin-containing CDDP albumin microspheres and CDDP solution. At a dose of 1 mg eq CDDP/kg microsphere A showed the highest platinum concentration at 1.3 μg/ml 1 h after administration; after peaking it gradually decreased, release was sustained and platinum concentration 7 d after administration was still 0.6 μg/ml. Microsphere B showed 0.8 μg/ml 1 h after administration, which was lower than microsphere A. Microsphere C showed 0.6 μg/ml 1 h after administration, then decreased and was 0.2 μg/ml 7 d after administration. CDDP solution was 2.6 μg/ml 1 h after...

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**Fig. 1.** Release Profiles of CDDP from Chitin-Containing CDDP Albumin Microspheres

Microsphere: ○, A (less than 20 μm); △, B (20–37 μm); □, C (37–74 μm).

**Fig. 2.** Plasma Concentration-Time Profiles of Platinum after Arterial Injections of Microsphere A, B, C and CDDP Solution

Values are mean ± S.D. (n=3). Microsphere: ○, A (less than 20 μm); △, B (20–37 μm); □, C (37–74 μm); ●, CDDP solution.
administration, then gradually decreased. The \( AUC \) value was \( 114.63 \pm 17.09, 83.72 \pm 10.14, 41.1 \pm 8.25, \) and \( 89.12 \pm 18.95 \text{h} \cdot \mu g/ml \) for microsphere A, B, C and CDDP solution, respectively. There were differences in \( AUC \) among the particle sizes.

Figure 3 shows the plasma concentration time course of platinum after administration of microsphere A and CDDP solution, at a dose of 1, 2 and 4 mg eq CDDP/kg. Plasma platinum concentration increased with increase of CDDP dose, and the increase in rate was different with CDDP microsphere and solution. The \( AUC \) value of microsphere A was \( 114.63 \pm 17.09, 136.01 \pm 13.15 \) and \( 150.96 \pm 15.96 \text{h} \cdot \mu g/ml \), for a dose of 1, 2 and 4 mg eq CDDP/kg, respectively, while the value of CDDP solution was \( 89.12 \pm 18.95, 112.62 \pm 14.95 \) and \( 129.41 \pm 21.87 \text{h} \cdot \mu g/ml \) for the same respective doses.

**Platinum Content in Hepatic Tissue** Table I shows platinum content in hepatic tissue at 7d after administration of microspheres A, B and C and CDDP solution. At a dose of 1 mg eq CDDP/kg hepatic tissue platinum content differed between the microsphere A, B and C group and the CDDP solution group. This content differed according to particle size with platinum content of microsphere A group being \( 3.43 \pm 0.52 \text{µg/g} \), B group \( 2.54 \pm 0.36 \text{µg/g} \), and C group \( 1.54 \pm 0.22 \text{µg/g} \). Microsphere A group was thus 2.2 times the platinum content of microsphere C group. The CDDP solution group was \( 2.91 \pm 0.63 \text{µg/g} \). There was no difference in platinum content between tumor and normal tissues in CDDP microspheres and solution administration \( (p>0.05) \).

Table II shows platinum content at 7d after administration of microsphere A and CDDP solution, at a dose of 1, 2 and 4 mg eq CDDP/kg. At a dose of 2 mg to the microsphere A group, the hepatic tissue platinum content was \( 4.84 \pm 0.75 \text{µg/g} \), about 1.8 times that obtained at 1 mg eq CDDP/kg. There was no difference in platinum content between tumor and normal tissues in CDDP microspheres and solution administration \( (p>0.05) \).

**Antitumor Effects** Table III shows the tumor growth rate in the rabbit VX2 hepatic carcinoma model. At a dose of 1.0 mg eq CDDP/kg. Tumor growth was increasingly suppressed with the decrease of particle size. The non-treated group showed remarkable tumor growth with a rate of \( 577.7 \pm 67.3\% \). In contrast, the growth rate of

**TABLE I. Effect of Particle Size on the Platinum Content in Liver**

<table>
<thead>
<tr>
<th>Microsphere</th>
<th>Tumor</th>
<th>Normal portion adjacent to tumor</th>
<th>Normal portion distant from tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.43±0.52</td>
<td>3.01±0.38</td>
<td>3.15±0.47</td>
</tr>
<tr>
<td>B</td>
<td>2.54±0.36</td>
<td>2.18±0.22</td>
<td>1.88±0.52</td>
</tr>
<tr>
<td>C</td>
<td>1.54±0.22</td>
<td>1.36±0.45</td>
<td>1.29±0.39</td>
</tr>
<tr>
<td>CDDP solution</td>
<td>2.91±0.63</td>
<td>2.61±0.57</td>
<td>2.72±0.43</td>
</tr>
</tbody>
</table>

\( n=3 \).

**TABLE II. Effect of CDDP Dose on the Platinum Content in Liver**

<table>
<thead>
<tr>
<th>Material</th>
<th>Dose (mg eq CDDP/kg)</th>
<th>CDDP concentration (µg Pt/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tumor</td>
</tr>
<tr>
<td>Microsphere A</td>
<td>1.0</td>
<td>3.43±0.52</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>4.84±0.75</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>6.12±0.66</td>
</tr>
<tr>
<td>CDDP solution</td>
<td>1.0</td>
<td>2.91±0.63</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>3.24±0.71</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>3.15±0.51</td>
</tr>
</tbody>
</table>

\( n=3 \).
TABLE III. Effect of Particle Size on Growth Rate of VX₂ Tumor in the Liver of Rabbits

<table>
<thead>
<tr>
<th>Microsphere</th>
<th>Growth rate % (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>90.3 ± 11.8³</td>
</tr>
<tr>
<td>B</td>
<td>101.8 ± 18.0²</td>
</tr>
<tr>
<td>C</td>
<td>176.4 ± 25.8</td>
</tr>
<tr>
<td>Microsphere⁴</td>
<td>378.3 ± 32.5</td>
</tr>
<tr>
<td>CDDP solution</td>
<td>246.3 ± 38.2²</td>
</tr>
<tr>
<td>Non-administration</td>
<td>577.7 ± 67.3⁵</td>
</tr>
</tbody>
</table>

p < 0.05 in A and B vs. C. Data from Refs. 14 and 15. n = 3.

TABLE IV. Effect of Dose on Growth Rate of VX₂ Tumor in the Liver of Rabbits

<table>
<thead>
<tr>
<th>Material</th>
<th>Dose (mg eq CDDP/kg)</th>
<th>Growth rate % (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsphere A</td>
<td>1.00</td>
<td>90.3 ± 11.8</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>70.6 ± 9.7</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>61.8 ± 10.8</td>
</tr>
<tr>
<td>CDDP solution</td>
<td>1.00</td>
<td>246.3 ± 38.2</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>220.5 ± 17.6</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>198.7 ± 20.3</td>
</tr>
</tbody>
</table>

p < 0.05 in a dose of 2 and 4 mg eq CDDP/kg vs. 1 mg eq CDDP/kg. n = 3.

the treated group without CDDP dropped to 378.3 ± 32.5%, and the chitin-containing CDDP microsphere group dropped to between 90.3 ± 11.8, 101.8 ± 18.0% and 176.4 ± 25.8%, at microsphere A, B and C, respectively.

Table IV shows the tumor growth rate in rabbits of microsphere A and CDDP solution measured under various dose conditions. The growth rate of microsphere A administration was 90.3 ± 11.8, 70.6 ± 9.7 and 61.8 ± 10.8% at a dose of 1, 2 and 4 mg eq CDDP/kg, respectively. Growth rate of CDDP solution administration was 246.3 ± 38.2, 220.5 ± 17.6 and 198.7 ± 20.3% at the same respective doses.

DISCUSSION

Chitin-containing CDDP albumin microspheres were prepared with various particle sizes, and effect of particle size on in vitro CDDP release, plasma platinum concentration and antitumor effect in VX₂ hepatic tumor model rabbits was examined.

In vitro, more CDDP release was observed with reduction in particle size. Plasma platinum concentration and AUC also differed with particle size.

Microsphere A showed the highest platinum concentration at 1.3 μg/ml 1 h after administration, and after peaking it gradually decreased until about 0.6 μg/ml was detected after 7d. These results indicated that, although the microspheres plugged arterioles in the liver, a portion of the microspheres was ingested by Kupffer’s cells or other reticuloendothelial cells by phagocytosis, and in the case of microsphere A, CDDP was rapidly released into blood.

Platinum content in the hepatic tissue was found to vary depending on the particle size, with tissue platinum content increasing as particle size decreased. This finding is considered to indicate that microspheres in smaller size are phagocytosed by the hepatic tissue and, as a result, are stored in the liver in higher quantities. The antitumor effect differed according to size of the particle, and the growth rate tended to be more greatly suppressed as particle size increased. However, no significant differences were observed between microspheres A and B. These observations could indicate that incomplete embolism or rapid metabolism of CDDP occurred in hepatic tissue, and therefore the effect of the size difference was not fully reflected. There has been a report that cancer cells themselves exhibit phagocytosis, and small particles are taken in by cancer cells by such mechanisms as endocytosis. On the basis of our results as well as the above-mentioned report, antitumor effects are considered to increase when the particle sizes of the microspheres are smaller. However, local necrosis in normal liver tissue was observed after administration of microsphere A. No marked histopathologic change was seen in any other microsphere group or the CDDP solution group. Therefore, administration of small particle size microspheres should be performed very carefully.

Using microsphere A, the relationship between the dose of CDDP and the changes in plasma platinum concentration, platinum content in hepatic tissue and antitumor effect were examined. Plasma platinum concentration and AUC were found to increase with the increase of CDDP dose. A similar increase was obtained in the analysis of CDDP content in hepatic tissue. With respect to antitumor effect, suppression of tumor growth rate was observed with higher doses, although no significant difference was observed between the doses of 2 and 4 mg eq CDDP/kg.

A technical consideration that may influence the accuracy of these results is the problem of back flow when a volume of material is injected into a restricted space (i.e. an artery of a small animal), or the difference in dose may not have been reflected fully in the growth-suppression due to rapid growth of the VX₂ tumor.

Our findings indicated that effective methods to increase antitumor activity of chitin-containing CDDP albumin microspheres would be to reduce particle size or increase the amount of CDDP dose. According to recent information, many cases of metastasis of hepatocellular carcinoma in the liver take place through the portal vein, and it is difficult to discriminate them from multicentric metastasis in with the liver. Considering the possibility of an incomplete embolism even with the increased doses used in the experiments, an effective route of administration of the spheres appears to be the portal vein as well as the hepatic artery.

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REFERENCES


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