Effects of Fluidity and Vesicle Size on Antitumor Activity and Myelosuppressive Activity of Liposomes Loaded with Daunorubicin

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The effects of fluidity and vesicle size on the antitumor activity and myelosuppressive activity of liposomes loaded with daunorubicin, an anthracycline antitumor drug, were investigated in Yoshida sarcoma-bearing rats. Liposomes composed of egg phosphatidylcholine (EPC) or hydrogenated egg phosphatidylcholine (HEPC), cholesterol and dicetyl phosphate in a molar ratio of 5:4:1 were injected intravenously into rats 5 days after subcutaneous inoculation of Yoshida sarcoma. At non-effect dosage in free drug, HEPC-liposomes with a diameter of 58 or 142 nm showed the greatest inhibitory effect against Yoshida sarcoma among liposomes tested, whereas larger ones (272 nm) had weaker effect. Small EPC-liposomes (57 nm) had no effect. Larger HEPC-liposomes (especially 142 nm) greatly decreased the number of peripheral white blood cell compared with free drug at the same dose, indicating relatively strong myelosuppressive toxicity. However, small EPC- and HEPC-liposomes with a diameter of 57 and 58 nm, respectively, showed toxic effects comparable to that of free drug. Examination of the dose-dependency of therapeutic effects and toxicity indicated encapsulation of daunorubicin in the small HEPC-liposomes to enhance the therapeutic index about 3 times that of free drug. These findings indicate the possibility of using small HEPC-liposome as a drug carrier for targeting solid tumors.

Keywords liposome; fluidity; vesicle size; tumor growth inhibition; myelosuppression; Yoshida sarcoma

Liposomes have been extensively studied as drug-targeting carriers to tumors.1–32 Liposomes injected into the blood rapidly disappear from the blood circulation due to their uptake by the reticuloendothelial system (RES) such as macrophages in the liver and spleen.33–35 This hinders their delivery to sites other than RES.36 Uptake by RES should be prevented for increased tumor accumulation of liposomes and for effectively treating tumor with liposomes containing antitumor agents.

A tumor has a characteristic morphology; the permeability of capillaries is enhanced compared with normal tissue and the lymphoid system is little developed.37 Thus, there is the possibility that small liposomes may extravasate more easily into extracellular spaces around tumors through the leaky capillary endothelium. Increasing the blood half-life of small liposomes by reducing RES uptake may augment the above possibility. Small liposomes59 or liposomes with rigid membranes60 tend to escape RES uptake. The inclusion of monosialoganglioside (GM1)7,11 or polyethylene glycol (PEG)12 in liposomes has been shown to effectively reduce the uptake of liposomes by RES and prolong their residence time in the blood.

Several groups have reported that small unilamellar vesicles (SUVs) with a diameter less than 100 nm may pass through the fenestration of liver sinusoids to be taken up by hepatic parenchymal cells.7,13 In a recent biodistribution study using Yoshida sarcoma-bearing rats, we found that 40-nm SUVs with rigid membranes composed of hydrogenated egg phosphatidylcholine (HEPC), cholesterol and dicetyl phosphate (5:4:1), exhibited greatly reduced liver uptake and high tumor accumulation compared with more fluid SUVs containing egg phosphatidylcholine (EPC) instead of HEPC.14,15 HEPC–SUV had the longest blood half-life, but was slightly inferior in tumor accumulation to 100-nm HEPC–liposomes whose accumula-

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suspensions were extruded through a polycarbonate membrane with a pore size of 0.4, 0.2 or 0.1 μm at least 10 times at 60 °C. The others were sonicated into small unilamellar vesicles (SUVs) and centrifuged at 105000 × g for 60 min to remove large particles and make a homogeneous size. Daunorubicin hydrochloride was dissolved in the liposomal suspensions. Each was neutralized with 5N NaOH and immediately incubated at 60 °C for 5 min. They were then placed in an ice bath. Unencapsulated drug was removed by dialysis against pH 7.4 phosphate buffered saline (Na2HPO4 1.15g, KH2PO4 0.2g, NaCl 8g, KCl 0.2g/l) at 5 °C. Drug encapsulation efficiency of the liposomes depended on the phosphatidylcholeline used or size of liposome and ranged from 50 to 100%. The final concentrations of total lipids and drug in liposomes were approximately 40 mm and 2 mg/ml, respectively. Drug concentration in each liposomal preparation was determined by the HPLC method after solubilizing liposomes with methanol. Vesicle size was determined by the dynamic light scattering with a submicron particle analyzer (Coulter N4 model, Coulter Electronics Inc., Hialeah, U.S.A.).

Animal Experiments Yoshida sarcoma cells (1 or 5 × 10⁶ cells) were inoculated subcutaneously into the right backs of male Domyu rats (5 weeks old, SLC, Shizuoka, Japan). Liposomal daunorubicin or free daunorubicin was injected via the tail vein into tumor-bearing rats on day 5. Control groups were treated with sterile saline. On day 9 or 10, after blood samples had been collected from the abdominal vein, tumors and spleens were excised and weighed. The numbers of blood cells (red blood cells (RBCs), white blood cells (WBCs) and platelets) were counted with an automated hematology analyzer (NE-6000, Sysmex, Kobe, Japan).

Statistics Following the F-test, statistical analysis was performed using the Student’s t-test, Aspin–Welch test or Cochran test. Differences were considered significant at p < 0.05.

RESULTS

Antitumor Activity of Liposomes Loaded with Daunorubicin EPC–SUV (57 ± 22 nm) and different sizes of HEPC–liposomes (58 ± 24, 142 ± 28 and 272 ± 100 nm) loaded with daunorubicin were injected intravenously into rats 5d after subcutaneous inoculation of Yoshida sarcoma (5 × 10⁵ cells). For accurate evaluation of drug-targeting effects, each liposomal drug was injected at a single dose, because multidose would possibly enhance the effect of free drug released from RES cells on antitumor activity. Figure 1 shows the results of tumor growth inhibitory tests of daunorubicin-containing liposomes. At noneffect dosage in free drug (4.32 mg/kg), all HEPC–liposomes significantly inhibited the growth of tumors. The effect observed for 58 or 142 nm of HEPC–liposomes was greatest. EPC–SUV had no effect.

**Myelosuppressive Activity of Liposomes Loaded with Daunorubicin** Table I shows the numbers of peripheral blood cells and spleen weights 5d after intravenous administration of liposomal preparations into Yoshida sarcoma-bearing rats at a dose of 4.32 mg/kg. Although the number of RBCs hardly changed by treatment with any preparation, that of WBCs or platelets decreased by treatment with larger HEPC–liposomes. Change in the number of WBCs was greater than that of platelets, and 142-nm HEPC–liposomes had severe toxic effect on WBC.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Liposome size (nm)</th>
<th>RBC × 10⁷/ml</th>
<th>WBC × 10⁷/ml</th>
<th>Platelet × 10⁷/ml</th>
<th>Spleen weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>7.74 ± 0.44</td>
<td>16.49 ± 2.48</td>
<td>10.32 ± 1.05</td>
<td>0.94 ± 0.18</td>
</tr>
<tr>
<td>Free drug</td>
<td></td>
<td>7.64 ± 0.39</td>
<td>10.44 ± 1.52</td>
<td>11.46 ± 1.76</td>
<td>0.60 ± 0.10</td>
</tr>
<tr>
<td>EPC–liposome</td>
<td>57 ± 22</td>
<td>7.28 ± 0.54</td>
<td>10.17 ± 3.34</td>
<td>9.96 ± 4.36</td>
<td>0.68 ± 0.16</td>
</tr>
<tr>
<td>HEPC–liposome</td>
<td>58 ± 23</td>
<td>7.60 ± 0.24</td>
<td>10.12 ± 2.57</td>
<td>9.14 ± 8.69</td>
<td>0.41 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>142 ± 28</td>
<td>6.73 ± 0.23</td>
<td>4.46 ± 0.34</td>
<td>8.04 ± 1.91</td>
<td>0.45 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>272 ± 100</td>
<td>7.75 ± 0.29</td>
<td>6.85 ± 2.30</td>
<td>6.22 ± 0.79</td>
<td>0.42 ± 0.11</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of Fluidity and Vesicle Size on Growth Inhibition Activity of Liposomes Loaded with Daunorubicin against Yoshida Sarcoma.

Daunorubicin-loaded EPC liposome (▪), HEPC liposome (▪), or free daunorubicin was injected intravenously into rats at a dose of 4.32 mg/kg on day 5 after subcutaneous inoculation of the tumor cells (5 × 10⁶ cells). Control rats were treated with sterile saline. Tumors were excised and weighed on day 10. Each bar represents the mean ± S.D. of 4–9 rats. The contents of DNR and lipids in each liposomal preparation were 2 mg/kg and 40 nm, respectively. a) p < 0.05, b) p < 0.001 for control, c) p < 0.001 for free drug, d) p < 0.05 for EPC liposome, e) p < 0.05 for 58-nm HEPC liposome, f) p < 0.01, g) p < 0.001 for 58-nm HEPC liposome, h) p < 0.001 for 58-nm HEPC liposome.

**Table I. Myelosuppressive Activity of Liposomes Loaded with Daunorubicin in Rats Bearing Yoshida Sarcoma**

Drugs were injected intravenously into rats at a dose of 4.32 mg/kg on day 5 after subcutaneous inoculation of the tumor cells (5 × 10⁶ cells). Control rats were treated with sterile saline. After blood samples were collected from the abdominal vein, tumors and spleens were excised on day 10. The values for blood cells and spleen weight represent the mean ± S.D. of 4–9 rats. The contents of DNR and lipids in each liposomal preparation were 2 mg/kg and 40 nm, respectively. a) p < 0.05, b) p < 0.01, c) p < 0.001 for control, d) p < 0.05, e) p < 0.01, f) p < 0.001 for free drug, g) p < 0.05 for EPC liposome, h) p < 0.05, i) p < 0.01, j) p < 0.001 for 58-nm HEPC liposome, k) p < 0.001 for 142-nm HEPC liposome.
However, HEPC–SUV (58 nm) and EPC–SUV (57 nm) showed toxic effects on WBC comparable to that of free drug and essentially no effect on platelets.

Loss in spleen weight was greater by treatment with HEPC–liposomes containing daunorubicin than free drug and was independent of vesicle size. For EPC–SUV containing daunorubicin, splenic weight loss was comparable to that shown by free drug (Table I).

Therapeutic Index of HEPC–SUV Loaded with Daunorubicin To determine the relation between antitumor effect and bone marrow toxicity of HEPC–SUV loaded with daunorubicin, we investigated the dose-dependency of antitumor effect or bone marrow toxicity in rats inoculated subcutaneously with Yoshida sarcoma (1 × 10³ cells) when the number of peripheral WBCs had decreased the most. Figure 2 shows change in the number of WBCs after intravenous administration of daunorubicin and daunorubicin-containing HEPC–SUVs (68 ± 21 nm) at various doses in Yoshida sarcoma-bearing rats. The decrease in WBC number induced by either preparation was unchanged from 3 to 5 d after intravenous administration, corresponding to 8 to 10 d after inoculation of tumor cells. WBC levels 3 to 5 d after intravenous administration of these preparations were judged to be the lowest or almost the lowest, because generally the number of peripheral WBCs reaches a nadir a few days after a single intravenous drug administration owing to the cells’ short half-life, and then gradually increases with recovery of lymphopoietic function.

Figure 3 shows dose–response curves of antitumor effect and toxicity on day 9. The curve of antitumor effect of HEPC–SUV loaded with daunorubicin shifted greatly to the left compared with free drug, while the curve of toxicity of the liposomal daunorubicin shifted slightly to the left. ED₅₀, TD₅₀ (toxic dose for 50% decrease of peripheral WBC number) and the therapeutic index of the liposomal daunorubicin estimated from the curves are summarized in Table II. ED₅₀ and TD₅₀ of the liposomal daunorubicin decreased 3.4-fold and 1.1-fold compared with free drug, respectively. The therapeutic index was about 3 times that of free drug, indicating enhanced usefulness of daunorubicin by encapsulation in HEPC–SUV.

DISCUSSION

The liposomes contained EPC or HEPC as “fluid” or “solid” phosphatidylycholine whose phase-transition temperature was about −10 or 54 °C, respectively. Daunorubicin is a lipophilic and amphiphilic compound and thus may affect fluidity and surface charge of the liposomal lipid bilayer. Each daunorubicin-containing liposome had almost the same ζ-potential and differential scanning calorimetry (DSC) curve as an empty liposome (data not shown). The pH gradient technique should make possible complete encapsulation of a drug in the inner phase of liposomes. The encapsulation of daunorubicin may thus have no effect on the original fluidity or surface charge of liposomal membranes.

The antitumor activity of liposomes containing an antitumor agent is thought to be related to tumor accumulation of liposomes as a carrier. The tumor accumulation of empty EPC–SUV (40 nm) has been shown to be intermediate between empty HEPC–SUV (40 nm) and HEPC–multilamellar vesicle (300 nm) in Yoshida sarcoma-bearing rats.¹⁴ However, EPC–SUV containing
daunorubicin had no antitumor effect, though approximately 300-nm HEPC-liposomes containing daunorubicin had weaker antitumor effect than HEPC–SUV containing daunorubicin (Fig. 1). EPC–SUV containing daunorubicin was unstable and most of the drug leaked quickly from the liposomes into the blood, whereas HEPC-liposomes containing daunorubicin were more stable in the blood (unpublished data). Thus, in EPC–SUV containing daunorubicin, not only antitumor activity but myelosuppressive activity may be the same as in free drug. Fluid liposomes are more sensitive to attack by plasma proteins than rigid ones and thus drug leakage from EPC–SUV may be facilitated by interaction with such proteins in the blood. Tumor accumulation of empty liposomes appears to be related more closely to that of HEPC–liposomes containing daunorubicin.

Recently, Liu et al. reported the optimal size for elevated tumor uptake of liposomes containing monosialoganglioside (GM1) to be from 90 to 200 nm. Empty HEPC–liposomes with a diameter of 100 nm were found to accumulate the most in a tumor. The size-dependency of tumor accumulation in GM1-containing liposomes or empty HEPC–liposomes is not always consistent with antitumor activity of daunorubicin encapsulated in HEPC–liposomes (Fig. 1). This suggests that the antitumor activity of liposomal antitumor drugs may not always be correlated with tumor accumulation of liposomes. A biodistribution experiment on these daunorubicin-containing liposomes in the same tumor model is in progress to elucidate this point. The reason why antitumor activity of 58-nm HEPC–SUV and 142-nm HEPC–liposome containing daunorubicin were almost the same may be explained as follows. First, small liposomes such as HEPC–SUV may be taken up more by tumor cells via endocytosis or pinocytosis which is enhanced in these cells. HEPC–SUV (59 nm) can be taken up by cultured Yoshida sarcoma cells in vitro via endocytosis at least 4 times more than larger HEPC–liposomes with a diameter of more than 100 nm. Second, liposomes situated in interstitial spaces of tumor may be local depots for drug release. This possibility is supported by microscopic studies of Papahadjopoulos et al. Since smaller liposomes have relatively loose packing among lipids in bilayers, they may show increased depot effect in tumor tissue. The encapsulation of an antitumor drug in liposomes has been reported to effectively reduce systemic toxicity and also toxicity in organs such as the heart or kidney due to the slow release of a drug from liposomes in blood circulation or reduced drug accumulation in these organs. Pharmacokinetic alternation by liposomes may not only reduce the toxicity of free drug, but increase toxicity in some organs or generate new toxicity.

Bone marrow is also considered a major site where liposomes localize after injection into the blood for the following reasons: 1) capillaries are fenestrated as in the liver and spleen; 2) bone marrow is abundant in phagocytic cells such as macrophages, monocytes and stem cells; and 3) wet weight of bone marrow is about 3 or 4% of body weight like that of liver in rats and human. Myelosuppression serves to greatly limit the dose of many antitumor agents. Myelosuppressive toxicity of 58-nm HEPC–SUVs containing daunorubicin was comparable to that of free drug, while that of 142-nm HEPC–liposomes containing daunorubicin was the greatest (Table I). Nevertheless, both liposomal preparations had maximal antitumor activity (Fig. 1). This suggests that: 1) myelosuppression of a drug may be increased by encapsulation into liposomes, 2) drugs entrapped in smaller liposomes may cause less myelosuppression, 3) the myelosuppressive activity of liposomal daunorubicin, as well as the antitumor activity, is regulated by liposomal size and 4) drug-containing HEPC–SUVs have better therapeutic activity than 142-nm liposomes.

Splenic damage may also decrease peripheral WBC number since the spleen is a lymphopoietic organ. As shown in Table I, splenic weight loss induced by daunorubicin-loaded HEPC–liposomes was greater than that induced by free drug and independent of vesicle size. This marked weight loss may be related to high accumulation of these liposomes in spleen where macrophages take up liposomes as in liver. However, leukopenia in 58-nm HEPC–liposomes was comparable to that in free drug. Therefore, splenic weight loss may contribute little to the decrease in WBC number.

Reasons worthy of consideration for the size-dependency of the toxic effect on WBC in HEPC–liposomes containing daunorubicin (Table I) are an abundance of phagocytic cells in bone marrow, slow blood-flow in bone compared with liver and the fact that larger liposomes can deliver greater quantities of trapped drugs to the marrow compartment. Larger liposomes are generally taken up more by macrophages due to their size. About 100-nm liposomes containing GM1 have been shown to be located exclusively in the resident macrophages of bone marrow. Low blood flow may cause prolonged retention time of liposomes, especially larger ones, in bone marrow. Thus, larger liposomes are likely to be taken up more by phagocytic cells and trapped drug in the liposomes would accumulate in these cells more than smaller liposomes. It has been suggested that pronounced and extended leukopenia in large (1.0 μm) doxorubicin-containing liposomes may be induced by drug leakage from marrow cells where they have accumulated. Daunorubicin may also leak from phagocytic cells in bone marrow and then redistribute in surrounding cells or tissue. This would be promoted by low blood flow in bones and strong affinity of daunorubicin for cells and/or tissue rather than for blood due to high lipophilicity.

The dependency of antitumor activity or myelosuppressive activity of liposomes loaded with daunorubicin on membrane fluidity and vesicle size (Fig. 1 and Table I) indicates that daunorubicin-containing HEPC–SUV, "solid and small" liposome, has the most therapeutic activity of the liposomes examined in this study. Detailed examination of the dose-dependency of therapeutic effects and toxicity showed the therapeutic index of daunorubicin encapsulated in HEPC–SUV to be about 3 times that of free drug (Table II). This indicates that the usefulness of daunorubicin is enhanced by encapsulation in HEPC–SUV.

Many attempts have been made to date to actively deliver antitumor agents to solid tumors via liposome
targeting. Unfortunately, most give reduced toxicity with maintenance of or reduction in antitumor effect. Thus, daunorubicin is considered to be effectively delivered to solid tumors (Yoshida sarcoma) and to show greater antitumor activity by HEPC–SUV. This means is thus seen as a potential drug carrier for passive targeting to solid tumors.

REFERENCES