Effects of Preparing and Ligand-Binding Methods of Small Unilamellar Liposomes on Their Blood Elimination and Tissue Distribution in Rats

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The effects of two methods of preparing small unilamellar vesicles (SUV) (detergent removal or sonication) on their in vivo elimination and tissue distribution was investigated in rats. The SUV prepared by either method had the same size distribution and lipid composition (egg yolk phosphatidylcholine/cholesterol/dipalmitoyl phosphatidylethanolamine or palmityc acid = 20/10/0.3, molar ratio). Three types of SUV made by either method were prepared. These contained one of three different surface ligand-binding functional groups (N-hydroxysuccinimide ester of palmitic acid, NHSP; glutaraldehyde-phosphatidylethanolamine, GA-PE; N-[4-(p-maleimidophenyl)butyryl]phosphatidylethanolamine, MPB-PE). SUV prepared by detergent removal were eliminated slowly from the circulation, and exhibited a low liver uptake and little leakage of [3H]insulin. There was no significant difference in elimination of the NHSP-SUV, GA-SUV or MPB-SUV prepared by detergent removal and their tissue distribution was similar. In contrast, the sonicated SUV were eliminated from the circulation much more rapidly mainly by liver uptake. The leakage of [3H]insulin from sonicated SUV into urine was relatively large. When sonicated control-SUV were prepared in the presence of the antioxidant, z-tocopherol (z-T-SUV), which reduces lipid peroxidation during sonication, the z-T-SUV were eliminated slowly with only a low liver uptake. Our results indicate that the rapid elimination and greater liver uptake of sonicated SUV is partly due to lipid peroxidation during preparation. These findings have relevance to the use of liposomes as a drug delivery system.

Keywords: small unilamellar liposome; elimination; tissue distribution; liver uptake; sonication; lipid peroxidation

Liposomes administered in the circulation are cleared by the reticuloendothelial system in the liver and spleen. The rate of liposome clearance depends on their size,3,4 lipid composition (especially cholesterol5–7), surface charge8,9 and surface ligand.10–12 Previously,13 we reported the clearance and tissue distribution in rats of dialyglycopetidetube-bearing small unilamellar vesicles (SUV) prepared by sonication. We observed that the elimination half-life of SUV prepared by sonication was shorter than that of SUV prepared by other methods. Although some studies have examined the effect on sonication on lipid peroxidation14–16 and the chemical instability of phospholipid in liposomes,17 there has been no investigation of the effect of sonication on the in vivo behavior of SUV.

In this study we investigated the effects of two different methods of preparation (sonication and detergent removal) and ligand-binding methods on the elimination of SUV from the circulation and tissue distribution in rats. Our results demonstrated significant differences in clearance and tissue distribution between the two methods of preparing SUV.

Materials and Methods

Materials

Egg yolk phosphatidylcholine (PC), synthetic dipalmitoyl phosphatidylethanolamine (PE), cholesterol (CH) and z-tocopherol (z-T) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Additional reagents were obtained as follows: glutaraldehyde, N-hydroxysuccinimide (NHS), palmitic acid, 2-mercaptoethanol, 2-thiobarbituric acid, 1,1,3,3-tetraethoxypropane and ethanolamine from Nacalai Tesque (Kyoto); egg yolk phosphatidylethanolamine hydrogenated (PCH) from Avanti Polar Lipids, Inc. (Pelham, AL, U.S.A.); succinimidyl-4-(p-maleimidophenyl)butyrate (SMBP) from Pierce Chemical Co. (Rockford, IL, U.S.A.); PL HQ-Auto 15 from Nissan Seiyaku Co. (Tokyo); [3H]insulin (308 μCi/mg) from New England Nuclear (Boston, MA, U.S.A.). All other reagents were commercial products of high purity and all operations utilized freshly redistilled water.

Preparation of Liposomes

N-Hydroxysuccinimide ester of palmitic acid (NHSP) incorporated SUV (NHSP-SUV) and glutaraldehyde-PE (GA-PE) incorporated SUV (GA-SUV), N-[4-(p-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE) incorporated SUV (MPB-SUV) were prepared to bind ligands, that possess a free amino group or a sulfhydryl group, on the outer surface of the liposomes. Ethanolamine or 2-mercaptoethanol was used as a model ligand. NHSP was synthesized according to the method of Lapidot et al.18 MPB-PE was synthesized according to the method of Martyn and Papadopoulous19 with some modifications. NHSP-SUV were prepared from 20 μmol of PC, 10 μmol of CH and 0.3 μmol of NHSP by each of the following two methods. In the detergent removal, sodium cholate (41 mg) was dissolved together with the lipids in 5 ml of chloroform:methanol (1:1) solution. After evaporation of the solvent, the dry lipid film was dispersed with 2 ml of phosphate-buffered saline (PBS, pH 7.4) containing 30 μCi of [3H]insulin, 1 mg of cold insulin. The suspension was incubated at 37°C for 5 min and dialyzed at 37°C for 4 h, then at room temperature overnight and finally at 4°C for another 24 h. Subsequently, the suspension was gel-filtered on a Sephadex 4B column. The collected liposomal fraction was mixed with 500 μmol of ethanolamine and incubated for 1 h. Uncoupled ethanolamine was removed by gel chromatography.

For sonication, lipids were dissolved in 5 ml of chloroform. After evaporation of solvent, the dry lipid film was dispersed with 2 ml of PBS containing 30 μCi of [3H]insulin and 1 mg of cold insulin. Sonication was sonicated in a probe-type sonicator (Tomy Seiko, UR-200R) for a total of 60 min (1 s sonication with 1 s cooling periods) at 0°C. The SUV formed were centrifuged at 3000 rpm to remove any metal powder, derived from the tip of the probe during sonication, then incubated with 500 μmol of ethanolamine for 1 h. Uncoupled ethanolamine was removed by gel chromatography. The SUV-SUV prepared by either detergent removal or by sonication were designated as NHSP-SUV(D) and NHSP-SUV(S), respectively.

GA-SUV were prepared from 20 μmol of PC, 10 μmol of CH and 0.3 μmol of PE using almost the same procedure described for NHSP-SUV. For detergent removal, 69 μg of sodium cholate was dissolved together with the lipids and the dry lipid film was dispersed at 70°C. The mixture was dialyzed at 70°C for 4 h, then at room temperature overnight. The SUV were treated with 50 μmol of glutaraldehyde for 30 min, then incubated with 500 μmol of ethanolamine for 1 h. For sonication, the sonicated SUV were treated with 50 μmol of glutaraldehyde for 30 min, then, incubated with 500 μmol of ethanolamine for 1 h. The GA-SUV prepared by either detergent removal or by sonication were designated as GA-SUV(D) and GA-SUV(S), respectively.

MPB-SUV were prepared from 20 μmol of PC, 10 μmol of CH and 0.3 μmol of MPB-PE using the procedure described for NHSP-SUV except for the replacement of ethanolamine with 2-mercaptoethanol (final concentration, 2%) in the blocking reaction. The MPB-SUV prepared by
either detergent removal or by the sonication were designated as MPB-SUV(D) and MPB-SUV(S), respectively. Three types of liposomes carrying covalently attached model ligand are illustrated in Chart 1.

Control-SUV were prepared from 20 μmol of PC, 10 μmol of CH and 0.3 μmol of PE using the procedure described for GA-SUV except for the treatment with glutaraldehyde and ethanolamine. The control-SUV prepared by either detergent removal or by sonication were designated as control-SUV(D) and control-SUV(S), respectively. The control-SUV(S) containing α-T were designated as α-T-SUV(S).

Size Distribution of Liposomes

The size distribution of [3H]ulin-containing SUV was estimated from that of cold SUV, prepared using the same procedure described above except for replacement of [3H]ulin withulin. Measurement of the size distribution of cold liposomes was carried out using an Autosizer, model 700 (Malvern, England). In Vivo Experiments and Determination of Radioactivity in Blood and Tissues

For the in vivo experiments, as previously described, except for the decoloration step, radioactivity in blood and tissue was also measured as previously described. Each 200 μl blood sample was added to 0.5 ml of H2O. Then 1 ml of 2N KOH-2-propanol solution (KOH: 2-propanol = 1:1) was added. The mixture was left to stand at room temperature overnight. The decolorized solution was neutralized with 1 ml of 10% CH3COOH and added to 10 ml of Scintisol EX-H. The mixture was left to stand in the dark for 6 h, and the radioactivity of samples was measured in a scintillation counter (Aloka, LSC-673). Tissue samples (0.2 g) were cut into fine pieces and transferred to a vial. Then, 0.5 ml of 30% H2O2 and 0.5 ml of 2N KOH-2-propanol solution were added and the mixture was incubated at 37°C overnight. Urine samples were diluted with PBS to a final volume of 3 ml, then 200 μl of diluted sample was transferred to a vial. To this sample, 0.5 ml of 2N KOH-2-propanol solution, 1 ml of 10% CH3COOH and 10 ml of Scintisol EX-H were added and mixed. Further procedures were carried out as those described for blood samples. Radioactivity in the circulation and in each organ were calculated and expressed as described before.

Lipid Peroxidation

The product of lipid peroxidation (malonaldehyde, MDA) during sonication was determined according to the method of Placer et al.20 with some modification. The 2-thiobarbituric acid (TBA) solution was prepared by dissolving TBA in a small amount of NaOH, then neutralized with 7% perchloric acid. The assay reagent was prepared by mixing 2 volumes of 0.8% TBA solution with 1 volume of 7% perchloric acid. The liposomal suspension (2 ml) containing PC or PCH(CHOH)2, 20 /mol with or without α-T was sonicated at 0°C in air. At set time intervals, 0.1 ml of the solution was sampled and mixed with 0.4 ml of 0.2 M Tris-HCl buffer (pH 7.4). To this mixture, 0.5 ml of assay reagent was added and heated in boiling water for 10 min. After cooling, 1 ml of pyridine - n-butanol (3:1, v/v) and 0.3 ml of 1N NaOH were added and mixed. Then, the mixture was centrifuged at 3000 rpm for 10 min and the absorbance of supernatant was measured at 548 nm. The standard MDA solution was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane with 0.1 N HCL at 40°C for 40 min. The calibration curve ranged from 1 x 10^-7 to 6 x 10^-5 M. Latency of the NHSP-SUV, GA- and MPB-SUV(S) in pooled rat plasma was measured as described before.

Results and Discussion

The size distribution of the liposomes is summarized in Table I. The average diameter of liposomes ranged from

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Average diameter ± S.E.(nm)</th>
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<tbody>
<tr>
<td>NHSP-SUV(S)</td>
<td>66.3 ± 22</td>
</tr>
<tr>
<td>NHSP-SUV(D)</td>
<td>101.4 ± 27</td>
</tr>
<tr>
<td>GA-SUV(S)</td>
<td>72.8 ± 25</td>
</tr>
<tr>
<td>GA-SUV(D)</td>
<td>75.7 ± 23</td>
</tr>
<tr>
<td>MPB-SUV(S)</td>
<td>103.1 ± 37</td>
</tr>
<tr>
<td>MPB-SUV(D)</td>
<td>103.9 ± 24</td>
</tr>
<tr>
<td>Control-SUV(S)</td>
<td>80.6 ± 28</td>
</tr>
<tr>
<td>Control-SUV(D)</td>
<td>109.2 ± 30</td>
</tr>
<tr>
<td>α-T-SUV(S)</td>
<td>96.9 ± 32</td>
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Fig. 1a. Clearance of NHSP-SUV(D), GA-SUV(D) and MPB-SUV (D) Prepared by Detergent Removal from the Circulation of Rats

Rats were injected intravenously with NHSP-SUV(D) (▲), GA-SUV(D) (●), MPB-SUV(D) (■) and control-SUV(D) (●). Each animal received 0.3 ml of [3H]ulin-containing liposome suspension (1 μmol of PC). Total radioactivity in the circulation was measured at the indicated time intervals and the results are expressed as a percentage of the injected dose. Each point represents the average of four animals ± S.E.

Fig. 1b. Tissue Distribution of NHSP-SUV(D), GA-SUV(D) and MPB-SUV(D)

At 3 h after the administration of 0.3 ml of [3H]ulin-containing liposome suspension (1 μmol of PC), rats were sacrificed and radioactivity in each organ was measured. The results are expressed as a percentage of the injected dose (■). NHP-SUV(D): □, GA-SUV(D): ●, MPB-SUV(D): ■, control-SUV(D): ▪. Each bar represents the average of four animals ± S.E. a) p < 0.05 compared to control-SUV(D).

66.3 ± 22 to 109.2 ± 30 nm. At these values, there was no difference in the average diameter of the different SUV prepared in this study. In addition, it is considered that the administered [3H]ulin-containing liposomes had essentially the same size distribution as cold liposomes. The elimination of NHSP-SUV(D), GA-SUV(D), MPB-SUV(D) and control-SUV(D) from the circulation is shown in Fig. 1a. The tissue distribution of these liposomes is shown in Fig. 1b. No significant difference was observed in
the clearance of the four types of liposomes prepared by detergent removal. Although the liver distributions of NHSP-SUV(S) and MPB-SUV(S) were less than that of control-SUV(D) \( (p < 0.05) \), the tissue distribution of these liposomes was similar. These findings indicate that the ligand-coupling method affects the elimination rate and the tissue distribution of liposomes prepared by detergent removal very little. The elimination of the NHSP-SUV(S), GA-SUV(S), MPB-SUV(S) and control-SUV(S) from the circulation is shown in Fig. 2a. The tissue distribution of these liposomes at 3 h post-injection and that of control-SUV(S) at 30 min post-injection are shown in Fig. 2b. The patterns of the elimination of these SUV exhibited two phases, phase I and II (before and after 30 min post-injection). The sonicated SUV were eliminated very rapidly in phase I, and slowly and linearly with almost the same rate (half-life, about 160 min) in phase II. The liver and urine distributions of \(^{3}H\)inulin at 30 min and 3 h post-injection of control-SUV(S) indicated that the rapid elimination of \(^{3}H\)inulin in phase I was ascribed mainly to the liver uptake of SUV, and that the slow elimination of \(^{3}H\)inulin from SUV. We obtained a similar blood clearance pattern when sialoglycopeptide-bearing SUV were administered in rats.\(^{13}\) NHSP-SUV(S) was eliminated faster than three other liposomes in phase I and the \(^{3}H\) level in urine reached 32% at 3 h post-injection. Since \(^{3}H\)inulin taken up an internalized into intracellular compartments is not readily metabolized and remains associated with tissues for a long period,\(^{22}\) and the free \(^{3}H\)inulin released from liposomes was cleared rapidly by the kidney and excreted into urine, the high value of \(^{3}H\) level in urine indicated the low latency of NHSP-SUV(S). We examined the latency of these three types of sonicated SUV in plasma using \(^{3}H\)inulin as a marker and found that the latency of NHSP-, GA- and MPB-SUV(S) at 3 h post-incubation was 80, 91 and 85%, respectively. The latency of NHSP-SUV(S) was however, not significantly lower than that of two other liposomes. The high value of \(^{3}H\) level in urine cannot be explained solely in terms of the obtained value of latency. Further studies are under way to investigate the cause for the high value of \(^{3}H\) level in urine.

Comparison of the clearance of liposomes prepared by the two methods revealed that SUV prepared by sonication were eliminated more rapidly than those prepared by detergent removal (control-SUV(D) in Fig. 1a and control-SUV(S) in Fig. 2a). It was also observed that the leakage of \(^{3}H\)inulin from SUV(D) was smaller than that of SUV(S). The \(^{3}H\) level in liver and urine at 3 h post-injection indicated that the increased clearance of SUV(S) was mainly due to liver uptake. Jana et al. reported\(^{14}\) that ultrasonic radiation induced lipid peroxidation in the liposomal membrane. It is conceivable that the increased liver uptake of SUV(S) was due to lipid peroxidation in the liposomal membrane.

To examine this possibility, we studied the effect of sonication on lipid peroxidation. Production of MDA during probe type sonication at 0°C in air is shown in Fig. 3. The control-SUV composed of PC, CH and PE (PC/CH/PE = 20/10/0.3, molar ratio) were used, since only PC having unsaturated acyl chain was considered to be peroxidized, whereas the ligand, ligand-binding functional groups, palmitoyl or dipalmitoyl chain were relatively stable during the sonication. Lipids in the liposomes were appreciably peroxidized when prepared without z-T. This peroxidation was slightly prevented by adding 0.5 mol% of z-T and strongly inhibited when 2 mol% of z-T was present in the liposomal lipids. Lipid peroxidation was completely inhibited when the liposomes were prepared with hydrogenated egg PC (PCH). These findings are consistent with those reported by Konings.\(^{16}\) We examined the effect of antioxidants on the clearance and tissue distribution of z-T-SUV(S). The elimination of z-T-SUV(S) from the circulation and their tissue distribution are shown in Figs.
the most significant difference was the more rapid in vivo elimination and greater liver uptake of the sonicated SUV compared to those prepared by detergent removal. Our results using α-T-SUV(S) indicate that the enhanced liver uptake of SUV(S) can be mainly attributed to lipid peroxidation during sonication. These results may be useful in the development and application of liposomes as drug carrier systems.

References and Notes
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