Pharmacodynamics and Tumoricidal Effect of Adriamycin Entrapped in Ceramide Sulfate-Containing Liposomes

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Received February 25, 1994; accepted May 24, 1994

Ceramide sulfate (N-acylsphingosine-1-O-sulfate), which lacks the galactose residue of sulfatide, was examined as a possibly preferable constituent of liposomes for drug delivery. Multilamellar vesicles prepared from phosphatidylcholine, cholesterol, and N-lignoceryl-N-acylsphingosine-1-O-sulfate in a molar ratio of 5:4:1 efficiently entrapped Adriamycin, and the retention of the drug in the liposomes in saline at 4°C for 8 days was nearly 100%. In terms of entrapment efficiency and retention of the drug in liposomes, N-lignoceryl-N-acylsphingosine-1-O-sulfate was superior to N-stearoylsphingosine-1-O-sulfate. A pharmacodynamic study revealed that the blood level of Adriamycin was far higher with the drug encapsulated in N-lignoceryl-N-acylsphingosine-1-O-sulfate-containing liposomes than with the free drug. The drug level in the heart was remarkably reduced with the liposome-entrapped drug, which is advantageous in reducing the cardiotoxicity of this drug. The effect of N-lignoceryl-N-acylsphingosine-1-O-sulfate-containing liposomes on the blood level of Adriamycin was superior to that of sulfatide-containing liposomes, though the effect of the former on the heart level was comparable to that of the latter. The tumoricidal effect on ascitic P388 leukemia and Lewis lung carcinoma was higher with Adriamycin entrapped in N-lignoceryl-N-acylsphingosine-1-O-sulfate-containing liposomes than with the free drug.

Keywords N-lignoceryl-N-acylsphingosine-1-O-sulfate; liposome; Adriamycin; tumoricidal effect; sulfatide; ceramide sulfate

In our earlier studies, we reported that sulfatide (CSE)-containing liposomes are promising drug carriers for cancer chemotherapy. For clinical application, liposomes are required to be stable in the blood and to retain a drug at a high level in the bloodstream for a long time. In a previous paper, we reported that both the negatively charged sulfatide group and the long acyl-chain of CSE contribute to the stabilization of liposomes, but not its galactose residue. Accordingly, we synthesized ceramide sulfate (N-acylsphingosine-1-O-sulfate, CERSE), which lacks the galactosyl moiety of CSE (Fig. 1), and found that liposomes containing it have high stability in phosphate-buffered saline (PBS), similarly to CSE-containing liposomes.

In the present study, we sought to obtain basic data on CERSE for its clinical application as a component of liposomes for the delivery of Adriamycin (ADM) as an anticancer agent. This paper reports the stability of CERSE-containing liposomes in terms of ADM retention, as well as the pharmacodynamics and tumoricidal effect of the liposome-entrapped ADM.

MATERIALS AND METHODS

Materials CERSE was synthesized as described previously. As fatty acid for the synthesis of CERSE, stearic and lignoceric acids were used; and two CERSEs, 1-O-sulfates of N-stearoylsphingosine (C18CERSE) and N-lignoceryl-N-acylsphingosine (C24CERSE), were obtained. CERSE was purchased from Pharmacia, Uppsala; egg phosphatidylcholine (PC), from Nippon Fine Chemical Co., Ltd., Tokyo; cholesterol (Chol.), from Sigma, St. Louis, MO; and ADM, from Kyowa Hakko Kogyo Company, Ltd., Tokyo. All other chemicals were of

Fig. 1. Structures of CSE and CERSE Sodium Salt

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analytical grade.

Preparation of Liposomes Liposomes entrapping ADM (40 mM lipids and 4.0 mM ADM) were prepared essentially as described previously.\(^\text{3}\) PC, Chol, and CSE or CERSE, in a molar ratio of 5:4:1, were dissolved in chloroform–methanol (2:1, v/v), and the solvent was evaporated with a rotary evaporator to form a thin film, which was then dried \textit{in vacuo} overnight. A solution of ADM in saline was added to the film and dispersed under a nitrogen atmosphere. The dispersion was sonicated at 20 kHz and 60 W by a probe-type sonicator (Model W-375, Heat Systems-Ultrasonics, Plainview, NY). Multilamellar vesicles (MLV) containing ADM were separated from small unilamellar vesicles (SUV) containing ADM and free ADM by passage through a column of Sepharose CL-2B (Pharmacia) as reported previously.\(^\text{1,2}\) SUV containing ADM were separated from MLV by centrifugation at 105,000 \(\times g\) for 1 h, and further separated from free ADM by Sepharose CL-6B chromatography. The size of CSE-, C18CERSE- or C24CERSE-MLV entrapping ADM was estimated by electron microscopy, and the distribution of 25–100 nm vesicles was found to be 65–74%. These vesicles are considered to be negatively charged from the data by electrophoresis reported previously.\(^\text{8}\) ADM concentration in the liposomes was determined as described by Kojima \textit{et al.}\(^\text{1}\) PC concentration in liposome suspensions was determined with a kit for PC (Phospholipids B-Test Wako, Wako Pure Chemical Industries, Ltd., Osaka).

Study on Pharmacodynamics Male ddY mice (6 weeks old) were injected with the drug (ADM entrapped in each type of liposomes or free ADM) at a dose of 5 mg/20 ml/kg body weight through a tail vein, and then sacrificed at 30 min, 1, and 2 h after injection. The amount of lipids injected as CSE-, C18CERSE-, or C24CERSE-liposomes was 72, 68, or 83 mg/kg body weight, respectively. Three mice were used for each test group. After collection of the blood by use of a heparinized syringe, the liver, kidney, heart, and spleen were excised. Tissues were rinsed with ice-cold saline, and the liver was further perfused with ice-cold saline. Determination of ADM concentration in the blood and tissues was carried out by the method described previously.\(^\text{1}\) Briefly, the tissues were homogenized with 5 volumes of water, and 0.2 ml of the homogenate was diluted with NaCl-saturated water to 2 ml. After the addition of 4 ml of n-butanol, the mixture was heated at 100 °C for 10 min and then vigorously shaken for 15 min. After centrifugation, the separated n-butanol phase was collected and its ADM concentration was determined fluorometrically at 590 nm with excitation at 470 nm.

Examination of Tumoricidal Effect The tumoricidal effect of free ADM or ADM entrapped in C24CERSE-containing liposomes was evaluated by use of mice bearing ascitic P388 leukemia or Lewis lung carcinoma. For estimation of the tumoricidal effect on P388 leukemia, male CDF1 mice weighing 25 to 27 g (6 weeks old) were inoculated intraperitoneally with \(1 \times 10^6\) cells of P388 leukemia, and at 24 h after the inoculation, the mice were injected intraperitoneally with the drug once. Five mice were used for each test group, and 10 mice for the control group. The mice of the test group were injected intraperitoneally with ADM in either free form or liposome-entrapped form at a dose of 5, 10, 15, 20, or 25 mg/kg body weight. Control mice were injected with saline. The survival time of the mice was recorded in days for 50 d after tumor inoculation. The antitumor effect was expressed as a percentage of the mean survival time of the test group to that of the control group. All mice that died were autopsied to assess lethal factors.

For estimation of the tumoricidal effect on Lewis lung carcinoma, female BDF1 mice weighing 17 to 22 g (6 weeks old) were implanted subcutaneously in their right flanks with \(2.5 \times 10^5\) cells of the carcinoma. Twelve mice were used in each group. ADM in either free or liposome-entrapped form at a dose of 2, 4, or 8 mg/kg body weight/d was injected intravenously into the mice through a tail vein three times, at 1, 5, and 9 d after tumor implantation. Control mice were given saline. The mice were sacrificed at 15 d after tumor implantation. Each tumor was excised, and its wet weight was measured.

RESULTS

Entrapment of ADM by Liposomes and Its Retention

The ratio of ADM/lipids in either the SUV or MLV of different compositions, and the retention of ADM in each type of liposomes after 8 days' storage in saline at 4 °C, are shown in Table I. The efficiency of entrapment of ADM was 51.0% for CSE-MLV, 52.8% for C18CERSE-MLV, and 49.2% for C24CERSE-MLV. Among the SUV tested, C24CERSE-containing SUV also showed an equivalent efficiency of entrapment to CSE-containing SUV. As shown in Table I, the leakage of ADM from liposomes in saline was smallest in C24CERSE-containing SUV. Whereas, C18CERSE-containing SUV showed the lowest values with regard to both the efficiency of entrapment of ADM and its retention. All of the MLV showed higher entrapment efficiency of ADM than the SUV. None of the MLV showed leakage of ADM from vesicles after the storage. The stability of liposomes in the serum was also examined by measuring the leakage of carboxyfluorescein (CF). Upon 120 min-incubation in 75% human serum, the retention of CF was 74% in the case of CSE-SUV (5:4:1) and 86% for C24CERSE-SUV (5:4:1), which had the most remarkable stabilizing effect, whereas PC/Chol-SUV (6:4) retained only 42% of CF. Accordingly, the following experiments were performed

<table>
<thead>
<tr>
<th>Table I.</th>
<th>Liposomes</th>
<th>Lipid composition (molar ratio, 5:4:1)</th>
<th>ADM/lipids (mol/mol)</th>
<th>Retention of ADM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUV</td>
<td>PC/Chol/CSE</td>
<td>0.025</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PC/Chol/C18CERSE</td>
<td>0.017</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PC/Chol/C24CERSE</td>
<td>0.023</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>MLV</td>
<td>PC/Chol/CSE</td>
<td>0.076</td>
<td>—100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PC/Chol/C18CERSE</td>
<td>0.077</td>
<td>—100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PC/Chol/C24CERSE</td>
<td>0.064</td>
<td>—100</td>
<td></td>
</tr>
</tbody>
</table>

Mean of 6 experimental data is given. Retention of ADM in liposomes is expressed as % of ADM retained in liposomes in saline after 8 d of storage at 4 °C. Mean of 2 experimental data is given.
with ADM entrapped in MLV.

**Pharmacodynamics of Liposome-Encapsulated ADM**

The amounts of ADM found in various tissues after the injection of ADM in different forms are represented in Fig. 2. When free ADM was injected, the amount of ADM in the bloodstream at 30 min after injection was minute. The highest blood concentration of ADM was maintained with 24CERSE-containing liposomes (24CERSE-MLV). The level of ADM in the blood was lower with 18CERSE-containing liposomes (18CERSE-MLV) than with 24CERSE-MLV. When ADM entrapped in CSE-containing liposomes (CSE-MLV) was injected, the blood level of ADM was much lower than that observed with either CERSE-MLV. The blood concentration of ADM at 2 h after the administration obtained with 24CERSE-MLV was 1.4, 11, and 94 times higher than than obtained with 18CERSE-MLV, CSE-MLV, and free ADM, respectively.

Entrapment of ADM by liposomes markedly reduced the level of ADM in the heart. The rate of decrease was almost the same for all the liposomes tested. In the pharmacodynamics of ADM in the liver, interesting drug clearances were observed. Free ADM showed gradual disappearance, probably due to either the excretion or degradation of ADM. In the case of CSE-MLV, the hepatic uptake of ADM was markedly increased, and a high level of ADM was maintained at 2 h after the injection. In contrast, the use of either CERSE-MLV significantly reduced the uptake by the liver and resulted in the same level as seen with free ADM at 2 h after the injection. Splenic ADM levels were significantly increased by the use of any of these liposomes. In contrast, the levels in the kidney were lower with all types of liposomes containing ADM than with free ADM.

**Tumoricidal Effect of 24CERSE-MLV-Entrapped ADM**

Since 24CERSE-MLV showed a high efficiency of ADM entrapment, low leakage of ADM from vesicles, and maintenance of a high blood level of ADM, we used this type of liposomes for the antitumor experiments.

In order to test the tumoricidal effect of ADM entrapped in 24CERSE-MLV, we carried out i.p. injection of the drug into mice bearing ascitic P388 leukemia and i.v. injection of the drug into those bearing Lewis lung carcinoma.

The tumoricidal effect of ADM entrapped in 24CERSE-MLV on P388 leukemia is summarized in Table II. The mean survival time of the control tumor-bearing mice was 10.1 ± 0.53 d after the tumor-cell inoculation. Upon single i.p. injection of the drug, free ADM appeared to be superior at the lowest dose (5 mg/kg body weight); and at this dose free ADM showed an increase in life-span (ILS) of more than 185%, while liposomal ADM showed an ILS of 90%. Mice injected with ADM entrapped in 24CERSE-MLV at a single dose of 10, 15 or 20 mg/kg showed over 200% ILS, but mice treated with the same doses of free ADM showed lower values. Mice treated with free ADM at a single dose of 20 mg/kg body weight showed 80% mortality by day 14. Mice treated at the highest dose of free ADM (25 mg/kg body weight) exhibited 100% mortality even before all control tumor-bearing mice died. By autopsy of these mice that died, we found the following pathological changes: peritonitis, fat necrosis throughout the abdominal cavity, adhesions of

![Graphs showing the pharmacodynamics of ADM in mice](image-url)

**Table II. Tumoricidal Effect on Mice Bearing Leukemia P388 of Free ADM or ADM Entrapped in 24CERSE-MLV**

| Samples | Dose (mg/kg) | Mean survival time (d) | ILS (%) | Long-term survivors b)
|---------|--------------|------------------------|---------|------------------------
| Saline  | —            | 10.1 ± 0.53            | —       | 0/10                   |
| Free ADM | 5            | >28.8 ± 8.73           | >185    | 2/5                    |
|         | 10           | 25.6 ± 4.28            | 153     | 0/5                    |
|         | 15           | 27.4 ± 4.25            | 171     | 0/5                    |
|         | 20           | >19.8 ± 7.59           | >96     | 1/5                    |
|         | 25           | 7.2 ± 0.97             | 29      | 0/5                    |
| Lip-ADM | 5            | 19.2 ± 2.85            | 90      | 0/5                    |
|         | 10           | >37.0 ± 7.96           | >266    | 3/5                    |
|         | 15           | 32.2 ± 7.37            | 219     | 2/5                    |
|         | 20           | >36.4 ± 7.35           | >280    | 3/5                    |
|         | 25           | >18.0 ± 8.31           | >78     | 1/5                    |

a) Increase in life span. b) This is defined as mice living for 50 d or more after inoculation of leukemia cells. Lip-ADM, ADM entrapped in 24CERSE-MLV. Mean ± S.E. is given. n = 10 for control group; n = 5 for each test group.
intraabdominal organs, and hemorrhage on the surface of organs, similar to a report by Litterst et al. It is clear that the cause of death of these mice was ADM toxicity but not tumor growth. At the same dose (25 mg/kg body weight), the use of C24CERSE-MLV reduced the toxicity of ADM and resulted in over 78% ILS.

When the number of mice that survived for more than 50 d after inoculation of the leukemia cells was compared, the total number of survivors that had been injected with ADM entrapped in C24CERSE-MLV was 3 times larger than that of those given free ADM.

Figure 3 demonstrates the tumoricidal effect of free or liposomal ADM on Lewis lung carcinoma implanted into female BDF1 mice. At a dose of 2 mg/kg body weight, neither free ADM nor liposomal ADM exhibited any antitumor activity. The tumors of mice administered ADM entrapped in C24CERSE-MLV at a dose of 4 mg/kg body weight were significantly smaller than those of the control mice (p < 0.05). At a dose of 8 mg/kg body weight, treatment with either free ADM or liposomal ADM brought about significant suppression of tumor growth, the significance of difference being p < 0.01 or p < 0.001, respectively. Liposomal ADM over the dose of 4 mg/kg body weight tended to exhibit a higher tumoricidal effect than free ADM, but no significant difference between them was observed.

DISCUSSION

Having a positive charge, ADM can interact electrostatically with acidic phospholipids. Several investigators demonstrated that negatively charged liposomes containing acidic phospholipids such as cardiolipin, phosphatidyserine, phosphatidylinositol, and phosphatidylglycerol were suitable as drug carriers of ADM. We have already shown that the ADM-entrapped efficiency of CSE-containing liposomes is higher than those containing other negatively charged phospholipids.

Concerning such liposomes, we wanted to know which part of the structure of CSE contributes to their stability. In a previous study, CERSE, lacking the β-galactosyl moiety of CSE, was synthesized, and CERSE having lignoceric acid as the acyl-chain was used as a liposome component. The resulting vesicles encapsulating CF showed increased stability in PBS and in serum. In the present study, C24CERSE-containing SUV showed a high degree of ADM entrapment, like CSE, and a remarkably low level of leakage of ADM, while C18CERSE-containing SUV did not. These results suggest that a long acyl-chain may rigidify the liposomal membrane by enhancement of the hydrophobic interaction. Further, we presently found that the MLV was superior to the SUV in terms of the entrapment and retention of ADM.

The fact that the blood concentration of ADM was higher with liposomal ADM than with free ADM is obviously ascribed to the entrapment of ADM, since CF entrapped in our liposomes was retained mostly in the liposomes after 2 h incubation in 75% human serum. The difference between the blood concentration of ADM obtained with C18CERSE- and C24CERSE-MLV at 2 h after injection and that obtained with CSE-MLV cannot be ascribed to the leakage of ADM from the liposomes but to the low uptake by the liver of CERSE-MLV compared to CSE-MLV. The uptake of liposomes may be due to receptor-mediated binding on the surface of hepatocytes. Gabizon and Papahadjipoulus reported that a higher concentration and longer blood residence time of liposomes were obtained by the insertion of CSE into the liposomes, and they suggested that the negatively charged sulfate group of CSE, situated on the equatorially oriented 3-OH group of galactose, may be sterically hindered by the sugar residue. In the present study, however, the blood concentration of ADM obtained with CERSE-MLV was higher than that with CSE-MLV, in spite of the fact that the negatively charged sulfate group of CERSE is exposed to the aqueous environment. Therefore, it seems that the high level of these liposomes in the blood is not due to the shielding of the negatively charged sulfate group but to the stability of these liposomes in the blood and their retarded clearance, the latter of which is probably due to the reduced hepatic uptake of these liposomes. It was demonstrated that β-galactosyl residue exposed on the outer surface of liposomes enhanced their uptake by the liver in vivo.

Among the data on pharmacodynamics, those on the heart are noteworthy regarding the clinical application of ADM. Since the deleterious side effects of ADM are mainly due to its toxicity to the heart, the reduced uptake of ADM by the heart is advantageous to decrease such side effects. In addition, it is noted that the uptake of CERSE-liposomes by the reticuloendothelial system was lower than that of CSE-liposomes.

When the treatments at the lowest dose of ADM were performed for the therapy of ascitic P388 leukemia, tumoricidal activity was higher with free ADM than with liposomal ADM. Probably the minimum effective dose of ADM in the abdominal cavity was not attained by the delay of the release of ADM from the liposomes. At higher doses of drug, however, i.p. administration of liposomal ADM showed a greater effect on ILS compared with that
of free ADM due to the suppression of the toxicity of ADM.

Although the effect of liposomal ADM on Lewis lung carcinoma was comparable to that of free ADM, the former is definitely superior than the latter because of the low toxicity mentioned above.

It is well known that tumors increase microvascular permeability21) and stimulate the formation of new capillary blood vessels penetrating the tumor.22) Accordingly, it may be that the great therapeutic efficiency of ADM entrapped in C24CERSE-MLV can be attributed to the maintenance of a high concentration of ADM in the bloodstream.

REFERENCES