Positively Charged Liposomes Containing Tumor Necrosis Factor in Solid Tumors

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The antitumor effects against solid tumors, such as Meth A sarcoma, MH-134 hepatoma and colon 26 adenocarcinoma, were examined after intratumoral administration of liposomes and Tumor Necrosis Factor (TNF) solution. The antitumor effects of liposomes against solid tumors were superior to those of TNF solution. In particular, the antitumor effect of positively charged (deacyl amine) liposomes was superior to that of negatively charged liposomes and TNF solution. Further, positively charged liposomes containing a higher dose of TNF than the solution could be administered without killing the mice, because of reduced side-effects. After intratumoral (Meth A sarcoma) administration, the TNF plasma concentration was determined in order to estimate the systemic side-effects of TNF. The area under curve (AUC) after administration of positively charged liposomes containing 6 times dose of TNF was about 1/30 the AUC after the administration of TNF solution. After administration of positively charged liposomes, TNF was mainly retained locally. Positively charged liposomes exhibited a stronger antitumor effect than the solution and had a lower AUC (about 1/180) than the solution. Consequently, some solid tumors could be completely cured by positively charged liposomes, because of their increased antitumor effect and reduced toxicity.

Key words TNF; positively charged liposome; deacyl amine; egg yolk; side-effect; antitumor effect

Tumor necrosis factor-alpha (TNF) exhibits potent antitumor activity in experimental studies on human tumor xenografts. However, in humans, administration of TNF is hampered by its severe systemic side-effects. The maximum tolerated dose ranges from 350 to 550 mg/m², which is at least 10-fold less than the effective dose in animals. Therefore, intravenous administration was rejected for the clinical testing of TNF, but local administration has been investigated in the hope of avoiding systemic side-effects. For local administration, not only direct injection into the tumor but also other methods, such as isolated organ perfusion and intraarterial injection, have been tried. Although these methods are also very effective in the case of a resectable localized cancer, the drug leaking from the organ to the systemic circulation causes systemic side-effects. Therefore, systemic side-effects are dose-limiting for TNF even in the case of intratumoral administration. Liposomes have been widely investigated for the delivery of antitumor drugs, but there are few reports about their local administration, since liposomes have been mainly used as a targeting device. Recently, cationic liposomes have been used for the introduction of DNA and RNA into tumor cells. Cationic liposomes adsorb on the cell surface and then introduce the drugs into the cells. In the case of intratumoral administration, TNF in the systemic circulation is not related to the antitumor effect, but to the side-effects. Therefore, we expected that positively charged liposomes would improve the amount of TNF retained locally, and exhibit an antitumor effect with reduced systemic circulation. The aim of this study was to confirm the potential of positively charged liposomes as a vehicle for local administration.

MATERIALS AND METHODS

Materials We purchased egg yolk phosphatidyl choline (PC), and egg yolk phosphatic acid (PA) from Nippon Fat and Oil Co., Ltd., and Decyl amine (DA) from Wako Pure Chemical Co., Ltd. The other reagents used were of analytical grade.

Preparation and Surface Charges of Liposomes In the case of PA liposomes, PC (70%) and PA (30%) were dissolved in chloroform while in the case of DA liposomes, PC (90%) and DA (10%) were dissolved in chloroform. The chloroform phase was evaporated in a round-bottom flask until a film was formed at the bottom of the flask. Subsequently, TNF solution was added to the flask with glass beads, and MLV liposomes were filtered through a 0.2 μm polycarbonate filter. Free TNF was separated on a Sepharose 4B column. These details were given in our reports. Particle size was determined by a laser scattering particle size analyzer, Coulter Nanosizer (Coulter Co., Ltd.). The surface charge of the liposomes was determined by light scattering equipment ELS-800 (Ohtsuka Electric Co., Ltd.). Liposomes were directly determined by enzyme immunoassay (EIA) without any treatment in order to determine the concentration of free TNF. The total content was determined by EIA after the addition of a detergent, Triton X-100. The properties of the liposomes used in this report are shown in Tables 1a and b. Liposomes were stable for a month at 4°C.

Release Tests These procedures were described in our reports. TNF assays were carried out by EIA. Free TNF was determined by EIA without any treatment, and total TNF was determined by EIA with Triton-X100. Various TNF concentrations were measured in standard samples to correct for the presence of detergent or plasma.

TNF Anti-tumor Activity in Vivo Female C3H, CDF1 and BALB/c mice were purchased from Shizuoka Laboratory Animal Center (Hama-matsu). The tumor cells used for transplantation were suspended in Eagle's minimum essential medium. A 0.1 ml aliquot of each tumor suspension (MH-134, 5×10⁶ cells/ml; colon 26, 2% brei; and Meth A, 2×10⁶ cells/ml) was transplanted i.d. into the abdominal wall of syngeneic female mice, 6–12 weeks old (C3H for MH-134; CDF1 for colon 26; BALB/c for Meth A). Mice with an ap...
appropriate size of tumor (6–10 mm diameter) were selected for experimentation 7 d after transplantation. A 0.1 ml aliquot of solution and liposomes was administered to groups of 7 mice, intratumorally. These procedures involving Meth A sarcoma are described in our reports. Blank liposomes were administered as a control in colon 26. The tumor volume was calculated from \(0.5 \times (W^2 \times L)\), where \(W\) and \(L\) represent the width (mm) and length (mm) of the tumor, respectively.

**Plasma Concentrations after Administration of Liposomes and Solution Containing TNF** When solid tumors, 6–10 mm in a diameter were formed, intratumoral administration was started by injecting liposomes or solution containing 60000 Japan reference units (JRU) and 10000 JRU (2.2 mg), respectively. Mice were sacrificed periodically and 0.3 ml aliquots of blood were collected. Blood samples were heparinized and centrifuged at 3000 rpm for 10 min. Then 0.1 ml of the plasma was collected. Plasma samples were frozen at \(-20^\circ C\) and stored until analyzed. TNF concentrations in plasma were determined by ElA and a detergent (TritonX-100) was added to samples to solubilize the liposomes. The percentage remaining TNF was calculated from the injected dose.

**RESULTS**

The antitumor effects of liposomes and TNF solution were evaluated using an MH-134 hepatoma (Fig. 1). As shown in Fig. 1, inhibition of MH-134 hepatoma growth after administration of solution was observed, but this was weaker than that after liposome administration.

In Fig. 1A, the difference in tumor growth between 30000 JRU/mouse and 60000 JRU/mouse was small. Further, a dose of 100000 JRU/mouse was also examined, but all mice died because of the side-effects of TNF solution. This indicates that the therapeutic range of TNF solution against MH-134 hepatoma is too narrow to produce a cure. On the other hand, a dose-dependence after liposome administration was observed (Figs. 1B and C), and 5 out of 7 tested mice after DA liposome (100000 JRU/mouse) administration were completely cured. Although 4 mice died after administration of solution (60000 JRU/mouse) because of side-effects, PA and DA liposomes (100000 JRU/mouse) could be administered because of the reduced side-effects. This reduced toxicity and increased effect demonstrates the usefulness of liposomal delivery, even after topical administration. A comparison of PA and DA liposomes revealed the superior antitumoral effect of DA liposomes, because the tumor growth inhibition produced by DA liposomes was more potent than that of PA liposomes after administration of the same dose. In this figure, the control group means "no treatment." At 12 d after administration, one mouse died and at 14 d another mouse died because of the tumor growth. The death of two mice with a large tumor volume resulted in a reduction in the

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**Table 1a. Characteristics of TNF Liposomes Immediately after Preparation**

<table>
<thead>
<tr>
<th>Abbreviation (A)</th>
<th>TNF Activities (10^4) (JRU/ml)</th>
<th>TNF latency (%)</th>
<th>Particle size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA liposome</td>
<td>455</td>
<td>96.3</td>
<td>188±66</td>
<td>-76.3</td>
</tr>
<tr>
<td>DA liposome</td>
<td>1,110</td>
<td>98.2</td>
<td>219±83</td>
<td>+63.8</td>
</tr>
</tbody>
</table>

**Table 1b. Characteristics of TNF Liposomes after 1 Month of Storage at 4°C**

<table>
<thead>
<tr>
<th>TNS Activities (10^4) (JRU/ml)</th>
<th>TNF latency (%)</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA liposome 413</td>
<td>96.8</td>
<td>188±73</td>
</tr>
<tr>
<td>DA liposome 1,190</td>
<td>99.2</td>
<td>217±55</td>
</tr>
</tbody>
</table>

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Fig. 1. Antitumor Effect of Solution (A), PA Liposomes (B) and DA Liposomes (C) against C3H Mice with MH-134 Hepatoma Transplants after Intratumoral Administration

The administered dose was 30000 JRU/mouse (■), 60000 JRU/mouse (▲) and 100000 JRU/mouse (×). Control (●) was "no treatment." Each value represents the mean±S.E. (n=7).

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tumor volume of the control group at 14 d.

Next, the antitumor effects of liposomes and TNF solution were evaluated in colon 26 adenocarcinoma (Figs. 2A, B and C).

The dose-dependence in the antitumor effects after administration of solution was confirmed in Fig. 2A. The antitumor effects after administration of solution and liposomes containing the same dose of TNF were very similar, but the solution (300000 JRU/mouse) could not be evaluated because of its toxicity. On the other hand, DA liposomes (300000 JRU/mouse) completely suppressed the tumor growth of colon 26 adenocarcinoma. The difference in antitumor effect between DA and PA liposomes was not very great, but PA liposomes (300000 JRU/mouse) could not completely suppress tumor growth. The antitumor effect against colon 26 adenocarcinoma was stronger than that against MH-134 hepatoma after administration of solution. At the same time, the lethal dose in mice with colon 26 adenocarcinoma transplants was less than that of mice with MH-134 transplants after administration of TNF solution. In spite of these advantages, colon 26 adenocarcinoma could not be cured by TNF solution and PA liposome administration. The reduced toxicity after DA liposome administration resulted in complete inhibition of colon 26 adenocarcinoma. Although the control group means "no treatment" in Fig. 1, each control group in this figure means a "vehicle" control (Figs. 2B and C) except for the solution (Fig. 2A). Tumor growth after administration of empty liposomes was almost the same as in the "no treatment" control, so empty liposomes do not have any antitumor effect. Therefore, the difference in antitumor effect between DA and PA liposomes was not due to the vehicles. In a similar manner, the difference in antitumor effect between the solution and liposomes was not due to the vehicles.

The antitumor activity of liposomes and TNF solution against mice with Meth A sarcoma transplants was examined (Fig. 3).

Liposomes and TNF solution containing the same dose of TNF exhibited different antitumor effects after intratumoral administration. The antitumor effect after administration of PA liposomes was the same as that after administration of TNF solution. Tumor growth in all mice was completely suppressed after administration of DA liposomes. Although no significant difference (more than 5%) between DA liposomes and TNF solution could be detected by Student's t-test, there was a tendency for the antitumor effect of DA liposomes to be greater than that of TNF solution.

Next, we studied the plasma concentrations in mice after the injection of these differently charged liposomes and TNF solution (Fig. 4).

After administration of TNF solution (10000 JRU/mouse), the time to reach the maximum peak plasma concentration ($T_{\text{max}}$) was very short and the maximum plasma concentra-
tion ($C_{\text{max}}$) was high. After administration of the same dose (10000 JRU/mouse) of liposomes, no TNF was detected in plasma. Therefore, 60000 JRU/mouse was administered in the case of liposomes. However, the $T_{\text{max}}$ was long and the $C_{\text{max}}$ was low after administration of PA liposomes. These findings suggest PA liposomes are able to release TNF in the body. On the other hand, after administration of DA liposomes, the TNF plasma concentration was very low. Although liposomes are biodegradable, this result suggests that DA liposomes are stable at least during this experiment.

Liposomes were incubated with a Tris–HCl buffered saline (pH 7.0) containing 0.1% bovine serum albumin (BSA) or 1 ml fresh heparinized rat plasma at 37 °C (Figs. 5a and b).

Both types of liposome did not release TNF in buffer during 60 min at 37 °C. In plasma, PA liposomes released TNF, showing that this release was induced by biochemical or biological factors, such as plasma components. This release can be triggered by Ca²⁺ ions in plasma. Although TNF was detected in plasma at 24 h after administration, plasma induced a rapid release of TNF. This phenomenon has been discussed in our previous reports. On the other hand, DA liposomes did not release TNF in plasma over a 60 min period. Although we do not have data for more than 60 min in plasma, DA liposomes did not release TNF during incubation in minimum essential medium with antibiotic agent for a period of 48 h. PA liposomes released TNF in this medium by the action of Ca²⁺ ions (Data not shown). Therefore, we believe that DA liposomes do not release TNF after intratumoral administration.

After intratumoral administration of TNF solution, locally retained TNF rapidly disappears. On the other hand, locally retained TNF after administration of liposomes was observed for 24 h (Fig. 6).

Generally, the barrier blocking diffusion from the injected site is the blood and lymph vessels, but liposomes cannot easily pass through vessels compared with TNF solution. Therefore, it was considered that a higher concentration of TNF at the injected site was observed after liposome administration. DA liposomes showed the highest concentration and this high TNF concentration resulted in a stronger antitumor effect of DA liposomes.

![Diagram](Fig. 4. TNF Concentration in Plasma after Intratumoral Administration of Solution (●, 10000 JRU/mouse), PA Liposomes (▲, 60000 JRU/mouse) and DA Liposomes (△, 60000 JRU/mouse)
Each value represents the mean ± S.E. (n=5).)

![Diagram](Fig. 5. TNF Release from PA Liposomes (●) and DA Liposomes (○) Incubated with Plasma (a) and Tris–HCl Buffered Saline (pH 7.0) Containing 0.1% BSA (b) at 37 °C)

![Diagram](Fig. 6. Locally Retained TNF (%) after an Intratumoral Administration of Solution (○), PA Liposomes (●) and DA Liposomes (△)
Each value represents the mean ± S.E. (n=5).

DISCUSSION

Even following local administration, the side-effects (death) were a dose-limiting factor of TNF in this study. There are few reports about intratumoral administration of liposomes containing antitumor drugs, since it has been considered that liposomes were not appropriate as vehicles for sustained release. Polylactic acid nanospheres and microspheres have been investigated as sustained release vehicles.

If release of TNF from DA liposomes occurred at the in-
jection site, TNF plasma concentrations should be detectable. Therefore, it was suggested that these positively charged liposomes directly act on tumor cells without the release of TNF. On the other hand, PA liposomes released TNF in plasma, and these liposomes showed a similar antitumor effect against transplanted tumors. The $C_{\text{max}}$ after intratumoral administration of PA liposomes containing TNF (60000 JRU/mouse) was lower than that after TNF solution (10000 JRU/mouse), and the $T_{\text{max}}$ was longer. Such sustained release from these negatively charged liposomes contributed to maintaining a high concentration of TNF at the injection site, and resulted in an adequate antitumor effect. Concerning the reduced side-effects after intratumoral administration of PA liposomes, the sustained release also contributed to suppressing the systemic circulation. Gradually released TNF from the injection site was rapidly cleared from plasma because of the non-linear elimination profile of TNF. As a result, it was possible to administer a higher dose than that in solution.

DA liposomes did not release TNF after intratumoral administration. Liposomes not releasing TNF do not exhibit any antitumor effect against Meth A sarcoma. The antitumor effect of DA liposomes is very strange. When DA liposomes were passed through a polycarbonate filter, a higher pressure is required than for other liposomes. Therefore, we considered that there are interactions between TNF and DA liposomes, and TNF is present inside liposomes. Further, there is little TNF outside liposomes, as shown by the EIA determination. DA liposomes exhibited the highest concentration at the injection site and the lowest plasma concentration after intratumoral administration. The highest concentration at the injection site contributed to the antitumor effect and the lowest plasma concentration contributed to the reduced side-effects. There are two targets for TNF to induce its antitumor effect. The first target is the tumor cells, themselves, and the second target is the blood vessels. It is our opinion that the positive charge on the liposomal surface interacts with the negative charge on the cell surface of tumor tissues and vessels. This interaction is one of the reasons for the strong antitumor effect and reduced side-effects of DA liposomes. However, the origin of the antitumor activity of DA liposomes cannot be proven at present. The area under the curve ($AUC$) after administration of DA liposome was lower (about 1/30) than that after administration of PA liposome. However, the lethal dose of TNF after administration of liposomes is very similar. For example, in mice with a colon 26 adenocarcinoma transplant, DA and PA liposomes (300000 JRU/mouse) killed one mouse within 24 h of administration. We believe that the lethal dose is too high to evaluate the side-effects of TNF, so that further studies are required.

In cationic liposomes, charged lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dimyristoyl-3-trimethylammonium propane (DMTAP) and dioctadecylamido-suberlipimine (DOGS) have been reported. These lipids were reported to be safe cationic lipids, compared with alkyl amines. In this study, decyl amine was used as a cationic lipid because it is easily obtainable. There is a possibility of further reducing the side-effects of TNF liposomes by using other cationic lipids, but additional studies are required. These findings are very interesting because of the potency of liposomal drug delivery with an adequate antitumor effect and reduced systemic circulation.

REFERENCES