Physiological Change after Local Injection of Liposomes Containing Tumor Necrosis Factor (TNF)

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After the local (subcutaneous) administration of tumor necrosis factor-alpha (TNF), two types of physiological change were clearly observed in dogs. One was a systemic change, the other was a local change at the injected site. Solution, negatively charged liposome and positively charged liposome were locally injected in dogs. Even with local administration, the increase of triglyceride in plasma and the decrease of blood pressure were the most serious after administration of the solution. These changes were typical systemic side effects of TNF. Consequently, liposomes suppressed these serious systemic side effects of TNF after a local administration. Another physiological change was irritation at the injected site. However, after administration of positively charged liposome, the lowest irritation at the injected site was observed, along with the highest local concentration of TNF. We reported the superior antitumor effect of positively charged liposomes in solution, as well as the lowest systemic circulation after an intratumor administration. Therefore, it was speculated that a positively charged liposome directly acted on the tumor cells without TNF release. These results exhibited the potency of liposomal delivery of TNF with local administration.

Key words liposome; tumor necrosis factor (TNF); physiological change; local administration; irritation; positively charged liposome

Liposomal drug delivery has progressed and its practical use was first employed with low molecular drugs. For example, such delivery of amphotericin B and doxorubicin were developed for clinical use. However, regarding high molecular drugs, liposomes have still not been developed for practical use. Further, liposomes have been researched as a delivery vehicle for targeting, but there are few reports about local administration. We report here that liposomes have potential as a delivery device for tumor necrosis factor (TNF) after local injection. TNF has a strong antitumor effect but also has serious toxicity, even if it is locally administered. Side effects have been one of the major obstacles to its therapeutic use. Common toxic side effects of TNF include fever, chills, rigor, fatigue, diarrhea, nausea, headache and hypotension, and severe hypotension, especially are dose limiting toxicity. Evaluation of these side effects was too difficult in tumor bearing mice and rats. For example, measurement of biochemical value requires a large blood sample, and rats and mice were too small to measure blood pressure. Although the potency of TNF liposomes in regards to efficacy and systemic circulation has been evaluated, the toxicity of TNF liposomes has not been evaluated. Positively charged liposomes exhibited a sufficient antitumor effect against tumor bearing mice and slight plasma concentration after intratumor administration. Low systemic circulation will be expected to be related to a reduction of such systemic toxicity. The potency of TNF liposomes after local administration will be discussed in this article.

MATERIALS AND METHODS

Materials We purchased egg yolk phosphatidyl choline (PC) from Nippon Fat and Oil Co., Ltd. and egg yolk phosphatidic acid (PA) from Nippon Seika Co., Ltd., according to our reports. We purchased decyl amine (DA) from Wako Pure Chemical Co., Ltd. Other reagents used were of analytical grade.

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Preparation and Surface Charges of Liposomes Preparation procedures of MLV liposomes were written in our report. Particle size was determined by a laser scattering particle size analyzer, Coulter Nanosizer (Coulter Co., Ltd.). Surface charges of the liposomes were determined by an electric light scattering device, ELS-800 (Ohkutsuka Electric Co., Ltd.). Characteristics of the liposomes used in this report are shown in Table 1. Liposomes were directly determined by enzyme immunoassay (EIA) without any treatment in order to determine the concentration of free TNF. Total content was determined by EIA after the addition of a detergent, Triton X-100. These liposomes were stable in the refrigerator for a month.

Biochemical Values After local (subcutaneous) injections of solution, PA liposome and DA liposome (200000 Japan reference units (JRJ)/kg), heparinized blood was collected periodically. Saline was also administered as a control. Sampled blood was centrifuged, and the plasma obtained was stored in a freezer until the determination of biochemical values; frozen plasma was thawed at room temperature. These values were determined using enzyme methods, except for glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). These measurements were determined by clinical test kits (Wako Pure Chemical Co., Ltd.). During measurement, dogs freely ate given foods and drank water.

Blood Pressure Each beagle dog was confined to an ex-

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>TNF activities ( \times 10^6 ) (JRJ/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>1110</td>
<td>98.2</td>
<td>219 ± 83</td>
<td>+63.8</td>
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clusive cage, and solution and liposomes containing TNF (200000 JRU/kg) were subcutaneously injected in the dogs. Blood pressures were measured on the leg of each dog using a manometer. Before the administration, unusual dogs were excluded by measuring blood pressures during 24 h.

**Locally Remaining TNF** Solution or liposomes containing TNF (10000 JRU/mouse) were subcutaneously administered to Balb/c mice. The mice were sacrificed periodically and subcutaneous tissue (about 10 mm×10 mm) was cut off. Samples were frozen at -20°C and stored until determination. Subcutaneous tissue was homogenized and centrifuged at 3000 rpm for 10 min. TNF concentrations in the tissues were determined by EIA, and a detergent (TritonX-100) was added to the samples. The percentage of remaining TNF was calculated against the injected dose.

**Irritations at the Injected Site** At 24 h after subcutaneous injection of the liposomes containing TNF (200000 JRU/kg), the conditions of the injected site were observed and photographed. At 24 h after injection, irritations were the most serious event. No special methods were used in this experiment.

**RESULTS**

**Biochemical Values** It was reported that positively charged liposomes exhibit a sufficient antitumor effect against tumor bearing mice and only slight plasma concentration after intratumor administration because of the high amount of liposome remaining locally. Biochemical values were investigated to determine physiological change after local injection. In this study, biochemical values were examined in normal dogs after subcutaneous injection, because of the difficulty of handling of tumor bearing animals.

Liposomes, TNF (200000 JRU/kg) or saline were locally injected at beagle dogs' backs. Blood was collected periodically, and the obtained plasma was measured to determine biochemical values. In this study, we determined the plasma concentration after administration, but we could not detect TNF in all samples.

After administration of a solution, biochemical values were obviously changed, except for total cholesterol (T. Cholesterol) compared to saline administration (p<0.05). Significant differences between solution and saline were observed in triglyceride (TG), GPT values and blood urea nitrogen.
(BUN). In creatinin and GOT values, significant differences were not observed, but an increasing tendency of these values was observed. These biochemical values indicated systemic physiological change caused by side effects of TNF. For example, an increase in TG value was a typical systemic side effect of TNF, and the solution clearly increased this value. For cancer patients, lack of TG are often observed, so this increase is a serious side effect. The increase of GOT and GPT values, especially GPT values, indicated hepatic injury of TNF after solution administration. The increase of creatinin and BUN indicated kidney injury of TNF after solution administration. These side effects were highest at 1 or 2 d after solution administration, and these changes were completely recovered at 7 or 9 d. In this experiment, a single administration of TNF was examined, but multiple administration is often applied to patients. Multiple administration will cause more serious side effects. In all values, liposomes suppressed these physiological changes. Therefore, liposomes are a useful vehicle for the suppression of this physiological change.

**Blood Pressure** As a next physiological change, blood pressure was examined in dogs. Severe hypotension is espe-

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**Fig. 2.** Systolic, Mean and Diastolic Blood Pressure after Subcutaneous Administration of Solution (●), PA Liposome (▲) and DA Liposome (△) Containing TNF (20000 JRU/kg) at Beagle Dogs' Backs

Each point represents the mean ± S.E. of 3 experiments. Statistical evaluation between solution and DA liposome was done by t-test. * p<0.05.

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**Fig. 3.** Locally Remaining TNF (%) after Subcutaneous Administration of Solution (●), PA Liposome (▲) and DA Liposome (△) Containing TNF (10000 JRU/kg) in Balb/c Mice

Each value represents the mean ± S.E. (n=3).
cially an dose-limiting factor of TNF. We did not examine the control group in this study, therefore, statistical evaluation was done between solution and DA liposome administered animals.

The subcutaneous injection of TNF solution caused a decrease in systolic, mean and diastolic blood pressure, but the DA liposome and PA liposome slightly affected these blood pressure parameters. Significant differences in blood pressures between solution and DA liposome administration were perceived by a t-test. Even with local injection, TNF solution led to serious hypotension. Especially, positively charged liposomes suppressed this physiological change. Therefore, as a potential vehicle, positively charged liposomes are useful regarding blood pressure.

Local Retention of TNF In our previous report, TNF remained locally after intratumor administration in mice. Locally remaining TNF after subcutaneous injection was determined in normal mice, owing to a comparison with intratumor injection.

Locally remaining TNF at the injected site after subcutaneous injection of solution also rapidly disappeared, as with intratumor administration. The disappearance of locally re-

Fig. 4. External Views at the Subcutaneous Injected Sites after PA Liposome (a) and DA Liposome (b,c) Containing TNF (200000 JRU/kg) in Healthy Beagle Dogs

These photographs were taken 24 h after administration. Illustrations of injected site for each photograph were attached, respectively.
remaining TNF after subcutaneous injection of liposomes was faster than that after intratumor injection. The value at 24 h after subcutaneous injection of DA liposome was about half that after intratumor injection. The value at 24 h after intratumor injection of PA liposome was 18.7%, but that after the subcutaneous injection of PA liposome could not be determined. These low local remainders suggest high systemic circulation. Although inferior in terms of locally remaining TNF after subcutaneous injection, as compared with intratumor injection, liposomes suppressed systemic physiological changes such as biochemical value and blood pressure.

Irritation at the Injected Site Even with local administration, TNF leaked to normal tissue led to irritation in humans. Tissue injury after local administration of TNF was the most prominent side effect in dogs. The extent of tissue injury was as follows, solution > PA liposome > DA liposome. Figure 4a shows typical damage in dogs in which PA liposome was administered. Hard bleeding and loss of hair were widely observed at the injected site. Swelling of injected site was not observed in this dog because of wide bleeding. Figure 4b shows the local irritation of the most seriously damaged dog in which DA liposome was administered. Slight bleeding, little loss of hair and slight swelling were observed at the injected site. Although it is difficult to recognize in this photograph, slight swelling at the injected site because of internal hemorrhage was observed. In another dog which was administered positively charged liposome, little bleeding, little hair loss and slight swelling were observed (Fig. 4c). Swelling is too difficult to be recognized in this photograph, once again.

There were large differences in the extent of swelling, but swelling of the injected site was observed in all dogs except for the hard bleeding dogs. These photographs demonstrate that PA liposomes cause more serious irritation than DA liposomes at the injected site. In this study, photographs of dogs after administration of solution were not exhibited, but the damage at the injected site was the most serious. Further, the most clear side effect we observed was in the appearance of these dogs, which were obviously weakened, after solution injection, by serious systemic side effects.

DISCUSSION

TNF produced severe side effects, even in the case of intratumor injection. These side effects are major obstacles for therapeutic use. Although we did not examine samples using endotoxin in this study, we considered that these serious side effects were not caused by contamination such as by endotoxin during preparation. If contamination caused these side effects, liposomes would have exhibited more serious side effects than solution. Liposomes require more complicated procedures to prepare, so liposomes have a much higher risk of contamination.

Subcutaneous injection to normal dogs as a substitution for intratumor injection to tumor bearing animals should be discussed for the evaluation of physiological changes. Mice and rats were too small to examine blood pressure and biochemical values. It was considered that subcutaneous injection produces weaker systemic side effects, because of higher local retention than intratumor injection. Tumor tissue has leaky blood vessels, so it was considered that systemic side effects such as changes in blood pressure and biochemical values after subcutaneous injection were smaller than those after intratumor injection. Therefore, it was considered that systemic side effects after subcutaneous injection must be observed after intratumor administration. On the other hand, it was too difficult to discriminate irritations from necrosis after intratumor injection. From these results, it was considered that subcutaneous injection to normal dogs were an appropriate substitution for intratumor injection to tumor bearing animals.

DA liposomes had superior antitumor effects, and DA liposomes also had the fewest side effects in this study. Although the mechanism of these effects of DA liposomes is still not sufficiently explained, it was revealed that DA liposomes did not release TNF at the injected site in this study. DA liposomes containing TNF showed higher viscosity than blank liposomes. Therefore, we considered that there are interactions between TNF and DA liposomes, but most of the TNF is inside of the liposomes, as shown by EIA determination. If there is a fair amount of TNF outside of the liposomes, the damage at the injected site must be more serious. This result suggested that DA liposomes act directly on cells or tissues without TNF release. Local irritation, for example bleeding and swelling, were visually similar to tumor necrosis, but the mechanism was quite different.

It has been reported that TNF induces phosphatidylycholine and sphingomyelin breakdown. These actions are related to the cytotoxic effect of TNF. In this experiment, liposomes suppressed systemic physiological changes such as fatty acid metabolism. These side effects also relate to the cytotoxic effect of TNF. Further, irritation at the injected site was suppressed by liposomal administration. Local irritation at the injected site was definitely a toxic side effect of TNF. This experiment indicated that liposomal administration of TNF selectively suppressed these side effects.

REFERENCES