Controlled Release by Ca^{2+}-Sensitive Recombinant Human Tumor Necrosis Factor-α Liposomes

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Recombinant human tumor necrosis factor-α (rHuTNF) was entrapped in liposomes consisting of Egg phosphatidylcholine (EggPC) alone, EggPC–egg phosphatidic acid (EggPA) or EggPC–egg phosphatidylglycerol (EggPG). These liposomes, stored in vials, were stable for a month at 4 °C. The rHuTNF release from the liposomes were examined in rat plasma and phosphate buffered saline (PBS). rHuTNF was released from the liposomes containing EggPA in rat plasma. The release of rHuTNF was inhibited by EDTA and was induced in PBS containing $CaCl_2$, indicating that this release is induced by Ca^{2+} ion in the plasma. The release of rHuTNF was promoted by an increase of the EggPA content.

In conclusion, we could obtain stable liposomes in a vial and this liposome could immediately release rHuTNF in the rat plasma. Furthermore we were able to control the release rate of rHuTNF from these liposomes.

Key words liposome; rHuTNF; calcium ion sensitive release; controlled release; stability; EggPA

In general, the drug entrapped in a carrier does not show any drug activities. Drug activity is observed after release from the carrier. Therefore, the control of drug release from the carrier is important.

The instability of the liposomes have always been a difficult obstacle in practical use, ever since Bangham discovered liposomes in 1965.[1] There have since been many reports about the improvement of liposomal stability,[2,3] but there are few reports about controlled-release of through liposomal membrane for drug release.

Recently, the stability of liposomes has been improved by freezing and freeze-drying.[4,5] Furthermore, the stability of liposomal solutions has also been improved, and recombinant human tumor necrosis factor-α (rHuTNF) liposomes stored at 4 °C for a month did not reveal any changes in this study. However, these stable liposomes were also stable in the body and did not show any antitumor activities.

Therefore, we examined the controlled release of the liposomes, and obtained sufficiently effective liposomes containing 30% egg phosphatidic acid (EggPA) liposomes *in vivo* with reduced side effect (data was not shown). Actually, egg phosphatidylcholine (EggPC) liposomes (3000 Japan Reference Unit (JRU)/mouse) did not show any antitumor effect *in vivo* (data was not shown). There have been some trials of drug release control from liposomes by the use of an external energy such as heat.[6,7] However these methods require the use of unusual apparatus.

The Ca^{2+} ion induced drug release from a nylon capsules with a phosphatidylethanolamine (PE) bilayer membrane.[8] We prepared and tested this PE liposome, but they were unstable and the release could not be the controlled efficiently.

Therefore, we used the interaction between an acidic phospholipid and divalent cations and prepared liposomes which have the following characteristics.

1. The liposomes don't release rHuTNF in the vial.
2. The liposomes do release their content in rat plasma.
3. The release rate from the liposomes can be controlled.

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Experimental

Materials EggPC was obtained from NOF Co., Ltd. and EggPA, egg phosphatidylglycerol (EggPG) were obtained from Nippon Fine Chemical Co., Ltd. All other reagents were used of analytical grade.

Preparation of Liposomes EggPC (1 g), EggPC (0.9 g)+EggPA (0.1 g), and EggPC (0.9 g)+EggPG (0.1 g) were dissolved in 10 ml of chloroform, and these solutions were evaporated until a film formed on the bottom of the flask. The drug solution (10 ml) was added to the flask and this flask was shaken with glass beads. These multi lamellar vesicle (MLV) liposomes were passed through the polycarbonate filter (0.2 μm) in order to control the liposomal size and the MLV liposomes were separated from the free drug by the gel permiation chromatography (Sepharose 4B).

This liposome, 1 ml, was poured into a vial filled with nitrogen and this vial was sealed.

Release Test This liposome, 100 μl, was mixed with 1 ml of fresh heparinized rat plasma or 5 mM Tris–HCl buffer (pH 7.0) containing various additives. This mixed solution was incubated at 37 °C and 50 μl of this mixed solution was sampled periodically and was immediately diluted with 5 mM phosphate buffered saline (PBS, pH 7.0) containing 0.1% bovine serum albumin. This diluted solution was directly determined by enzyme immunoassay (EIA) without any treatment in order to determine the concentration of released rHuTNF from the liposomes. That total content was determined by EIA after the addition of detergent, triton X-100.[9]

Particle Size Particle size was determined by a laser scattering particle counter, Coulter Nanosizer (Coulter Co., Ltd.). Liposomes, 100 μl, were sampled and diluted with 5 mM PBS (pH 7.0). The particle size was determined and each value was shown as the mean and standard deviation.

Results

The addition of charged substances to the liposomal membrane suppresses aggregation and leakage of the content, thus maintaining liposomes stability.[10] Therefore, rHuTNF liposomes containing EggPA or EggPG or 100% EggPC liposomes were prepared, and stability tested. Table I shows the lipid compositions of the liposomes and the content, entrapped ratio and particle size determined immediately after preparation and after 1 month of storage at 4 °C.

The particle size of the EggPC liposomes did not change greatly after storage. However the precipitation of liposomes in the vials was observed. This precipitation could be easily resuspended by shaking. In these

TABLE I. Lipid Composition in rHuTNF Liposome Preparations and Characteristics of rHuTNF Liposomes Immediately (A) after Preparation, and Characteristics of rHuTNF Liposomes after 1 Month Storage at 4 °C (B)

Abbreviations	EggPC	EggPA	EggPG	A			B		
				rHuTNF activities $\times 10^3$ (JRU/ml)	rHuTNF latency (%)	Particle size (nm)	rHuTNF activities $\times 10^3$ (JRU/ml)	rHuTNF latency (%)	Particle size (nm)
10% EggPA	900 mg	100 mg		1510	97.3	162±60	1431	97.5	168±60
20% EggPA	800 mg	200 mg		905	95.8	191±70	918	94.1	196±72
30% EggPA	700 mg	300 mg		455	96.3	186±66	413	96.8	188±73
10% EggPG	900 mg		100 mg	1520	98.8	206±72	1535	99.1	175±66
100% EggPC	1000 mg			1160	97.9	220±83	1138	94.6	215±72

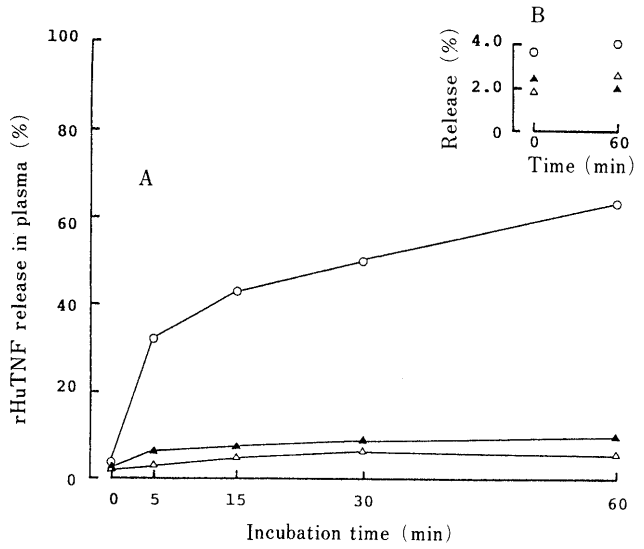

Fig. 1. rHuTNF Release from Liposomes after Incubation with Rat Plasma (A) or 5 mM Buffer (pH 7.0) Containing 0.1% BSA (B) at 37 °C

These liposomes were composed of EggPC: EggPA = 9 : 1 (○), EggPC: EggPG = 9 : 1 (△) and EggPC = 10 (▲).

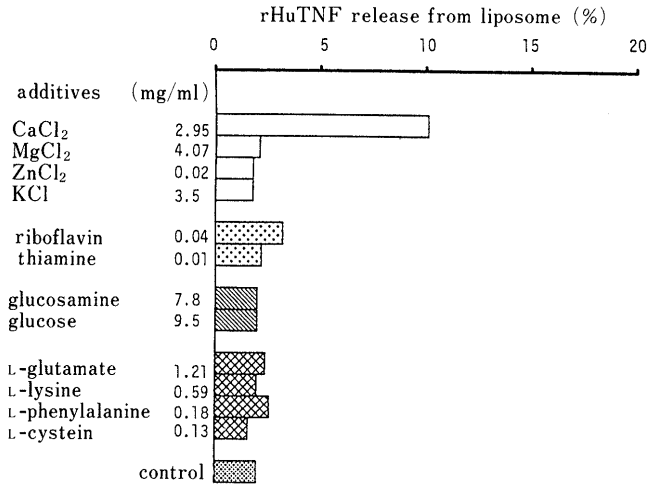

Fig. 2. rHuTNF Release from Liposomes after Incubation with 5 mM Buffer (pH 7.0) Containing 0.1% BSA and Various Additives for 30 min at 37 °C

This liposome was composed of EggPC: EggPA = 9 : 1.

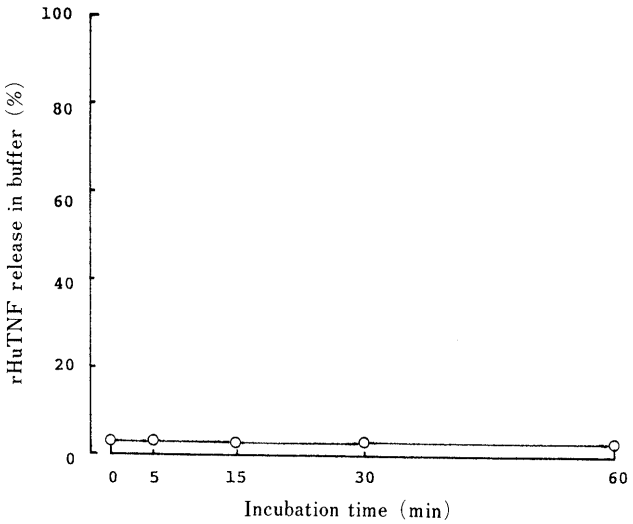

Fig. 3. rHuTNF Release from Liposomes after Incubation with Buffer Containing $CaCl_2$ (2.95 mg/ml) at 37 °C

This liposome was composed of EggPC: EggPG = 9 : 1.

experiments, these liposomes were stable during storage at 4 °C.

Next, these liposomes were incubated with rat plasma. rHuTNF was released only from the liposomes containing EggPA, in rat plasma (Fig. 1A). On the other hand, rHuTNF was not released in PBS (Fig. 1B).

The EggPG liposome composed of an acidic phospholipid was incubated in PBS containing $CaCl_2$, but rHuTNF release was not observed. The release of rHuTNF was not caused by dilution of the liposomes, because the isoosmotic pressure was maintained during these experiments. Furthermore, it is apparent that the release of rHuTNF was not caused by heat, because no release was observed in PBS at the same temperature as in rat plasma.

Therefore, subsequently, EggPA liposomes were incubated in PBS containing various biological components (Fig. 2).

In these experiments, each concentration of component component was decided by referencing to each endogenous plasma concentration. rHuTNF release was only observed in the PBS containing $CaCl_2$. By contrast, rHuTNF release was not observed in PBS containing $MgCl_2$ which also has a divalent cation.

These findings suggested that the Ca^{2+} ion induced the rHuTNF release from the EggPA liposomes. The EggPG is also minus charged phospholipid. Ther fore, we tried to examined the release of rHuTNF liposome in buffer. However, EggPG liposome did not release rHuTNF in a buffer containing $CaCl_2$ (Fig. 3).

To test the possibility of enzymatic membrane damage,

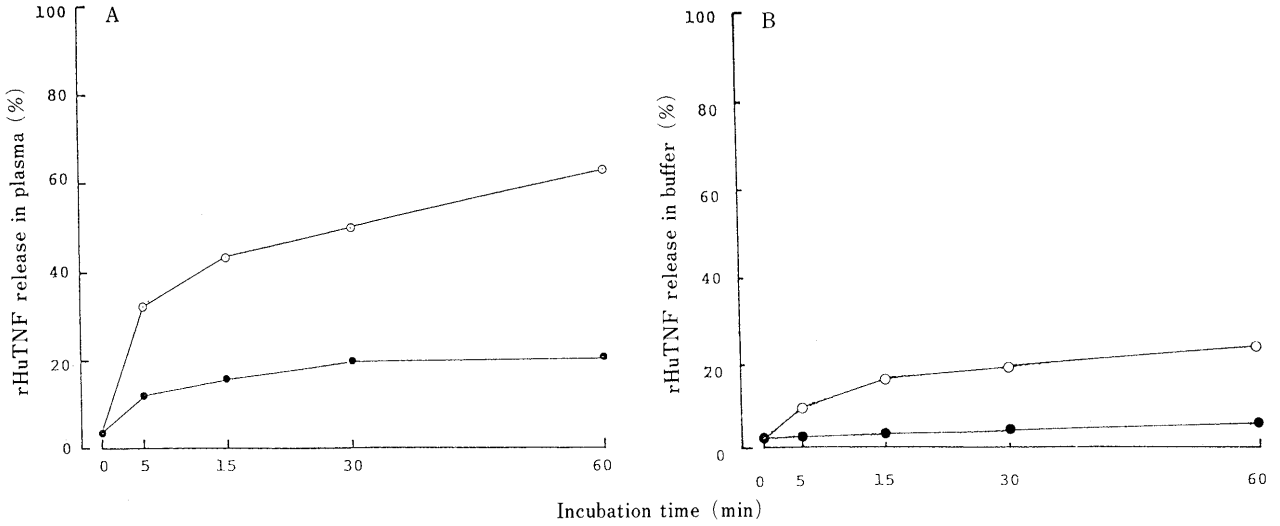

Fig. 4. rHuTNF Release from Liposomes after Incubation with Rat Plasma (A) and 5 mM Buffer Containing $CaCl_2$ (2.95 mg/ml) (B) at 37 °C (○) or 4 °C (●)

This liposome was composed of EggPC : EggPA = 9 : 1.

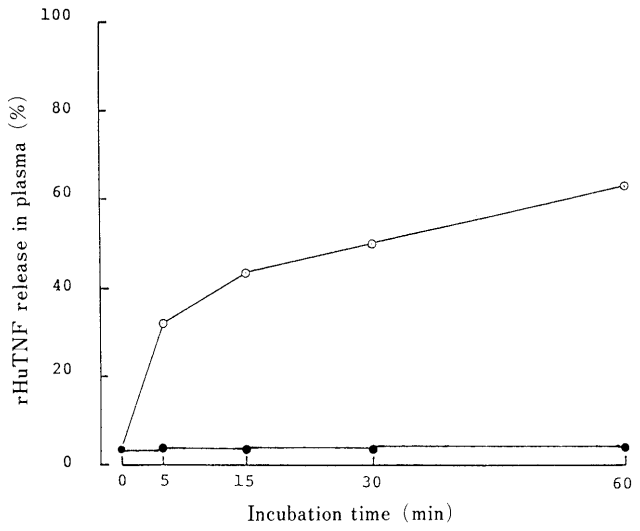

Fig. 5. rHuTNF Liposomes after Incubation with Rat Plasma Containing 10 mM EDTA (○) or no EDTA (●) at 37 °C

This liposome was composed of EggPC : EggPA = 9 : 1.

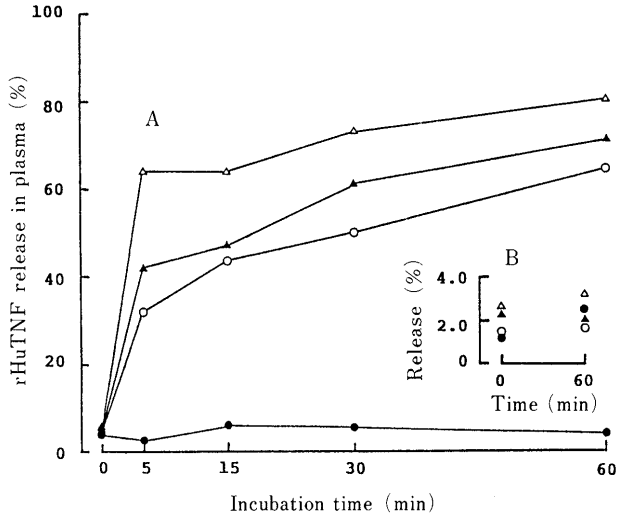

Fig. 6. rHuTNF Release from Liposomes after Incubation with Rat Plasma (A) or 5 mM of Buffer (pH 7.0) Containing 0.1% BSA (B) at 37 °C

These liposomes were composed of EggPC : EggPA = 10 : 0 (●), 9 : 1 (○), 8 : 2 (▲) and 7 : 3 (△).

EggPA liposomes were incubated with rat plasma at 4 °C and 37 °C, respectively. rHuTNF release was suppressed at 4 °C (Fig. 4A).

However, this suppression was also observed in PBS containing $CaCl_2$ (Fig. 4B).

Furthermore, this rHuTNF release in rat plasma was inhibited by the addition of EDTA (Fig. 5).

Next, we tried to control the rHuTNF release from the liposomes by changing the lipid component. The liposomes containing 20 and 30% EggPA were prepared, and the rHuTNF release from the liposomes was examined in rat plasma (Fig. 6A) and in PBS (Fig. 6B).

According to the increase in EggPA content, the rHuTNF release rate from the liposomes increased.

Discussion

In this study, the rHuTNF release from the EggPA liposomes in the rat plasma was induced by the Ca^{2+} ion. This release has the following characteristics: First, the Ca^{2+} ion induced the release, but the Mg^{2+} ion did not. Secondly, EggPA liposomes released rHuTNF, but EggPC and EggPG liposomes did not.

EDTA suppression probably did not occurr by an enzymatic reaction. Caffrey *et al.* reported that the liquid crystalline phase transition temperature of an EggPC–EggPA system was from 8 to 20 °C on the basis of the X-ray diffraction data.[11] Therefore, the liposomal membrane was in a crystalline state at 4 °C, and thus suppression may have occurred.

The lamellar structure of the liposomes changes into a hexagonal (HII) phase as a result of the interaction with a divalent cations.[12] However this change is also induced by the Mg^{2+} and the Mn^{2+} ions, there being no specificity for the Ca^{2+} ions.

In nature, the contents of the cell are released with a Ca^{2+} high sensitivity. In a number of processes related to exocytosis or secretion, there is a high specificity for the

Ca^{2+} ion.[13] Mei-June Liao *et al.* reported that membrane fusion of the EggPA liposomes is induced by the Ca^{2+} ion, while the Mg^{2+} and the Ba^{2+} ions show lower efficiencies.[14] Sundler *et al.* reported that PA liposomes fuse in the presence of the Ca^{2+} and the Mg^{2+} in the threshold concentration range of 0.03—0.1 mM (Ca^{2+}) and 0.07—0.15 mM (Mg^{2+}), respectively.[15] We cannot explain the rapid release of content from the rHuTNF liposomes with high Ca^{2+} specificity. Therefore, further study about this mechanism is required.

Next, EggPA did not interacted with a divalent cations. Interactions between the other acidic phospholipids and divalent cations have also been reported; for example, PS,[16] PG[17] and PI.[15] PA dissociates to PA^- and PA^{2-}, while, PG, PS and PI are monovalent. However, the PK_1 and PK_2 of PA are, 3.5 and 8.5, respectively.[18,19] There is negligibly little PA^{2-} in pH 7.0 PBS, so that PA is also monovalent in this study.

Papahadjopoulos *et al.* reported that PA liposomes fuse in the presence of at a concentration higher than 0.2 mM Ca^{2+} ion, and PG liposomes fuse at a concentration higher than 10 mM.[20] This suggests again that membrane fusion induces the release of rHuTNF from the EggPA liposomes.

In conclusion, we could obtain a Ca^{2+}-sensitive liposome which is stable in a vial and released rHuTNF relatively immediately in rat plasma.

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