Rates of Systemic Degradation and Reticuloendothelial System Uptake of Calcein in the Dipalmitoylphosphatidylcholine Liposomes with Soybean-Derived Sterols in Mice

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The systemic degradation and the reticuloendothelial system (RES) uptake of calcein entrapped in dipalmitoylphosphatidylcholine (DPPC) liposomes with soybean-derived sterols (SS) were examined after intravenous administration to mice by measuring the free and liposomal calcein levels in the blood. The results indicate that the rates of systemic degradation and the RES uptake of liposomes decrease with the addition of SS in DPPC liposomes since the SS has the ability to stabilize the liposomes. The rate of uptake by RES is larger than the rate of systemic degradation. The rate of leakage of calcein from liposomes by incubation in plasma *in vitro* is almost the same as that of systemic degradation *in vivo*.

KEY WORDS: dipalmitoylphosphatidylcholine (DPPC) liposomes; soybean-derived sterols (SS); systemic degradation; reticuloendothelial system (RES) uptake.

INTRODUCTION

Liposomes have demonstrated considerable promise as a carrier for the delivery of drugs *in vivo*. When administered intravenously, liposomes generally tend to be taken up by the reticuloendothelial system (RES) existing in the liver and spleen, and even when RES uptake can be avoided, they tend to be decomposed in the systemic circulation (1).

Recently, we demonstrated that dipalmitoylphosphatidylcholine (DPPC) liposomes with soybean-derived sterols (SS) were very stable compared to only DPPC liposomes *in vitro* (2) and *in vivo* (3). Especially, when the molar ratio of DPPC and SS was 7:4, the liposomes have the greatest stability, and the area under the concentration-time curve (AUC) was dramatically higher than those of liposomes with different DPPC and SS molar ratios (3).

In this study, we evaluated the rates of systemic degradation and RES uptake of DPPC liposomes with SS in order to clarify the difference in AUC among the liposomes of DPPC/SS, by measuring the free and liposomal calcein levels in the blood when calcein entrapped liposomes were administered to mice.

MATERIALS AND METHODS

Preparation

The preparation methods of DPPC liposomes with SS were described elsewhere (3). Four types of liposomes were prepared with 35 μmol DPPC and various molar ratios of SS [liposomes of DPPC/SS (7:0; 7:2; 7:4; 7:7)] by a reverse phase evaporation vesicle method. As a marker, 1ml of 20 mM calcein in 1/10 dilution of phosphate-buffered saline in distilled water (pH = 7.31, PBS) was used in the preparation. DPPC was purchased from Nippon Oil & Fats Co., Ltd. (Tokyo, Japan). SS used in this study was a mixture of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%) and was kindly provided by Ryukakusan Co., Ltd. (Tokyo, Japan). Calcein was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals used were of reagent grade. Liposomes were successively extruded through polycarbonate membranes (Nuclepore, U.S.A.) of 1.0, 0.4 and 0.2 μm pore size at about 60 °C. Unencapsulated calcein was removed by gel filtration of the liposome suspension through a Sephadex G-50 column (1.8 × 35 cm; Pharmacia, Sweden) with the PBS in all fractions. The concentration of DPPC in the liposomes was determined by an enzymatic assay using a Phospholipid B-test Wako (Wako Pure Chemical Industries, Ltd. Osaka, Japan). The amount of calcein entrapped in the liposomes was determined using a fluorescence spectrometer (excitation at 490 nm and emission at 520 nm; Hitachi F-4010, Tokyo, Japan). The captured volume was 8.04 ± 0.15, 7.08 ± 0.07, 10.8 ± 2.4, 12.7 ± 2.5 L/mol lipid for the liposomes of DPPC/SS (7:0, 7:2, 7:4, 7:7), respectively. The average diameter for each type of liposomes was approximately 0.2 μm after extrusion through the 0.2 μm pores of polycarbonate membranes, respectively (3).

Animal Experiments

Male ddY mice weighing about 30 g (7-weeks old) purchased from Saitama Experimental Animal Supply (Saitama, Japan) were used in all experiments (n = 3). Free calcein and the calcein entrapped in liposomes of DPPC/SS were injected via the tail vein at a dose of 2.5 μmol/kg weight of calcein, and then a 10-μl blood sample was obtained from the tail vein. The determination of the amount of calcein in the blood as described previously (3). The free calcein levels (C_{free}) and the total calcein levels (C_{total}) in the blood were determined before and after the addition of Triton X-100, where C_{free} expresses the free calcein released from the liposomes before the addition of Triton X-100, and C_{total} expresses the total calcein that was released from the liposomes and still entrapped in the liposomes in the blood. The liposomal calcein levels (C_{lipo}) in the blood were calculated according to ($C_{lipo} = C_{total} - C_{free}$). The total blood content was assumed to be 7.3% of the body weight of mice and the calcein level in the blood was expressed as a percentage of the injected dose.

Clearance Kinetics for Liposomes

The systemic elimination of liposomes after intravenous administration can be explained by RES uptake and systemic degradation of the liposomes (Fig. 1). The liposomes after intravenous administration immediately enter the systemic circulation and mix with the systemic blood. During

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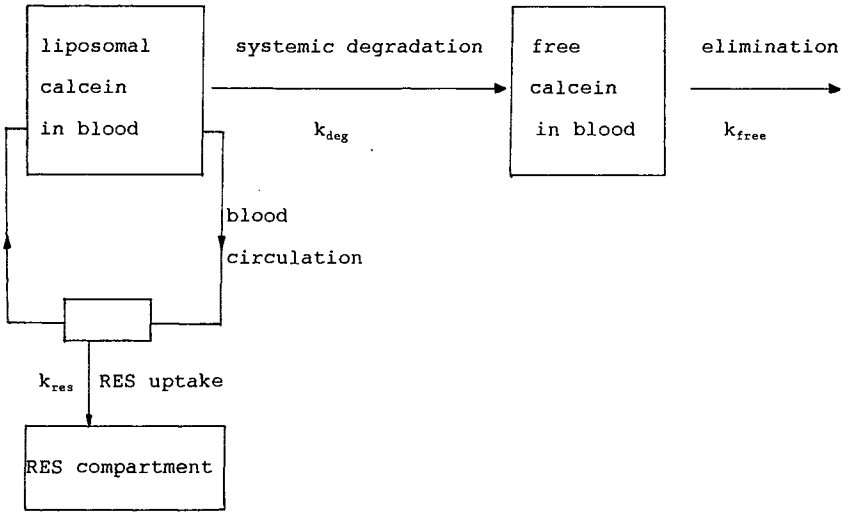

Fig. 1. The kinetics model of liposome elimination after intravenous administration.

circulation, some of the liposomes are degraded therein, while others are taken up by the RES when they pass through the RES compartment (4).

According to a clearance theory (4-6), the average rate of systemic elimination of liposomes (k_{lipo}) can be expressed as:

$$k_{lipo} = [100 - C_{lipo}(t)] / AUC_{lipo}(t) \quad (1)$$

where $C_{lipo}(t)$ refers to the liposomal calcein level in the blood at time t and $AUC_{lipo}(t)$ refers to the area under the curve of C_{lipo} levels between time 0 and time t.

According to the model of Fig. 1, the free calcein level in blood at time t ($C_{free}(t)$) can be expressed as:

$$dC_{free}(t)/dt = k_{deg} \cdot C_{lipo}(t) - k_{free} \cdot C_{free}(t) \quad (2)$$

where k_{deg} and k_{free} refer to the average rates of systemic degradation of liposomes and elimination of free calcein, respectively. We assumed that k_{lipo} is only determined by the rates of the RES uptake (k_{res}) and k_{deg}, k_{lipo} is the sum of k_{res} and k_{deg}, and therefore, k_{res} can be estimated from k_{lipo} and k_{deg} (4):

$$k_{res} = k_{lipo} - k_{deg} \quad (3)$$

where we assumed that liposomes distributed in the RES do not release the entrapped calcein back into the systemic circulation.

RESULTS AND DISCUSSION

Systemic Clearance of Free and Entrapped Calcein After Administration of Liposomes

After injection of free calcein solution, the free calcein was rapidly eliminated at a first-order rate from the blood (Fig. 2). The k_{free} was 0.031 ± 0.008 min^{-1} and is assumed to be constant during the elimination of free calcein from the blood after administration of both calcein solution and liposomes.

The C_{total}, C_{lipo} and C_{free} levels after administration of the liposomes are shown in Fig. 3. After administration of the liposomes without SS, [DPPC/SS (7:0)], the C_{lipo} levels were lower than the corresponding C_{free} level and the liposomal calcein was eliminated from the blood 30 min after administration. In contrast, the liposomes with SS, [DPPC/SS (7:2, 7:4, 7:7)], the C_{lipo} levels were much higher than the corresponding C_{free} levels. The C_{lipo} levels are higher in the following order: DPPC/SS (7:4) > DPPC/SS (7:7) > DPPC/SS (7:2) > DPPC/SS (7:0)-liposomes.

Rate of Systemic Degradation of Liposomes

We can estimate k_{deg} by using Eq. 2 as shown in Fig. 4. The k_{deg} (0.196 min^{-1}) of the liposomes of DPPC/SS (7:0) was 1.6-2.1 times higher than those of the liposomes of DPPC/SS (7:2, 7:4, 7:7) at 5 min and rapidly decreased to zero at 15 min. The k_{deg} of the liposomes with SS decreased from 0.095-0.127 min^{-1}, respectively, to constant values. The k_{deg} (0.004-0.006 min^{-1}) of liposomes of DPPC/SS (7:2, 7:4, 7:7) were similar to that (0.3 hr^{-1}) of DPPC liposomes with disteroylphosphatidylcholine (7:3, w/w) studied by Iga and co-workers (4). These results suggest that the liposomes of DPPC/SS (7:0) were unstable and rapidly degraded in blood due to the interaction of the bilayer of the liposomes

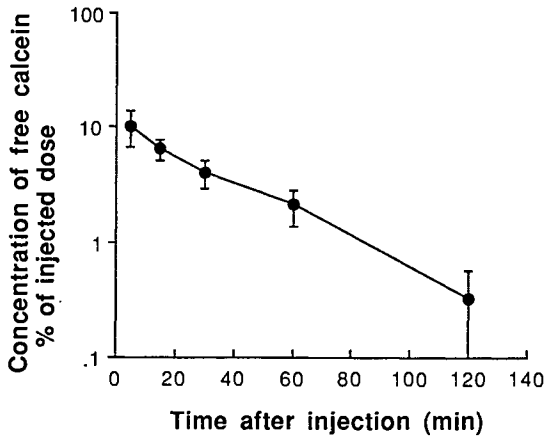

Fig. 2. Time-course of systemic clearance of free calcein after administration of calcein solution. Values are means±S.D. (n=3).

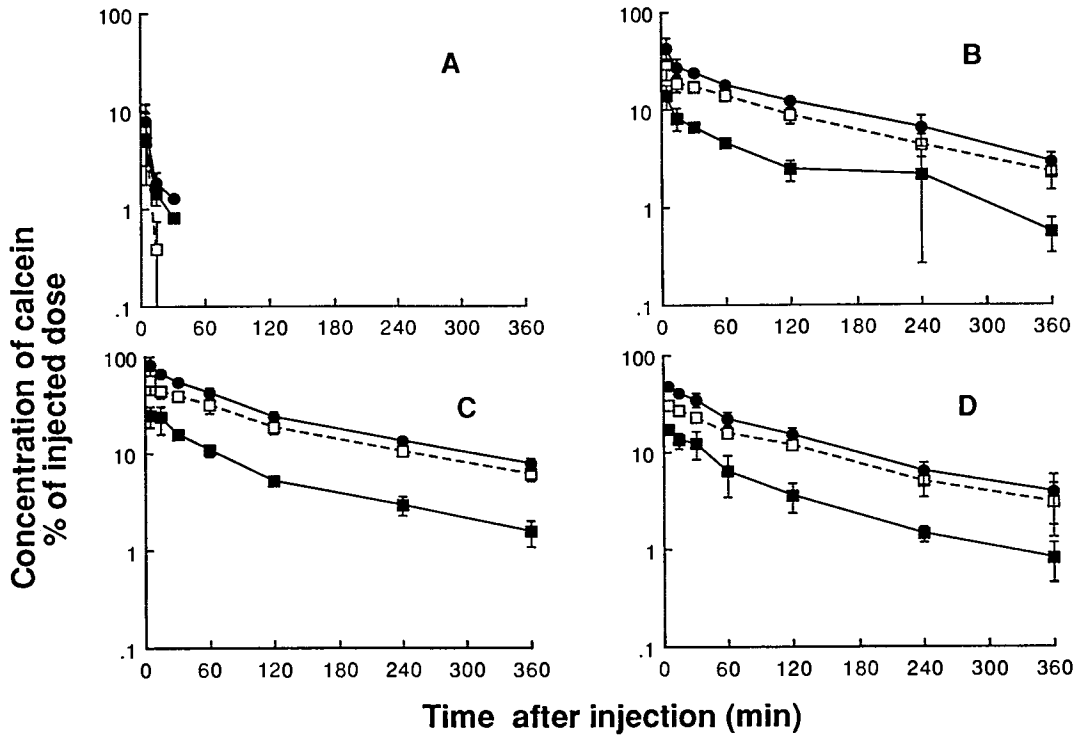

Fig. 3. Time-course of systemic clearance of free calcein (—■—), liposomal calcein (---□---) and total calcein (—●—) after administration of liposomes. (A) is DPPC/SS (7:0)-liposomes, (B) is DPPC/SS (7:2)-liposomes, (C) is DPPC/SS (7:4)-liposomes, (D) is DPPC/SS (7:7)-liposomes. Values are mean±S.D. (n=3).

with the proteins in blood, and SS has the ability to stabilize the bilayer of the DPPC liposomes (2, 3).

Rate of RES Uptake of Liposomes

The k_{res} can be estimated by using k_{deg} and k_{lipo} in Eq. 3, and is shown in Fig. 5. The k_{res} of the liposomes of DPPC/SS (7:0) was 15.0, 26.3, and 11.2 times that of liposomes of DPPC/SS (7:2, 7:4, 7:7) at 15 min after administration of the liposomes, respectively. These results suggest that the uptake by RES mainly occurred during the early stage after administration, similar to the result reported by Iga and coworkers (4). The liposomes of DPPC/SS (7:4) have the lowest k_{res} among the liposomes of DPPC/SS. These results

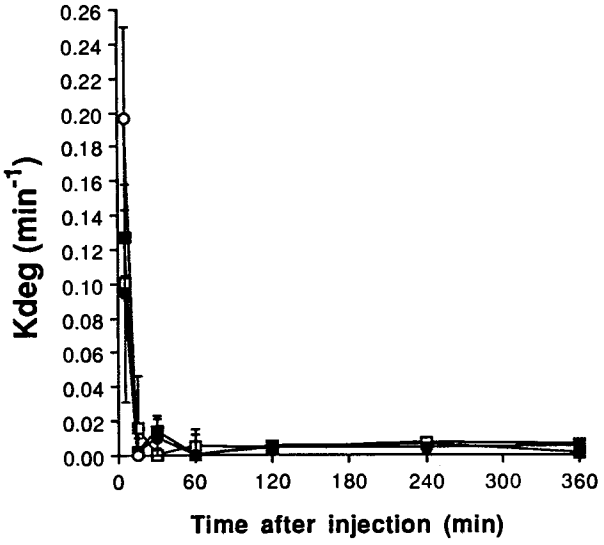

Fig. 4. k_{deg}-time profiles after administration of liposomes. (■) is DPPC/SS (7:7)-liposomes, (□) is DPPC/SS (7:4)-liposomes, (●) is DPPC/SS (7:2)-liposomes, (○) is DPPC/SS (7:0)-liposomes. Values are mean ± SD (n = 3).

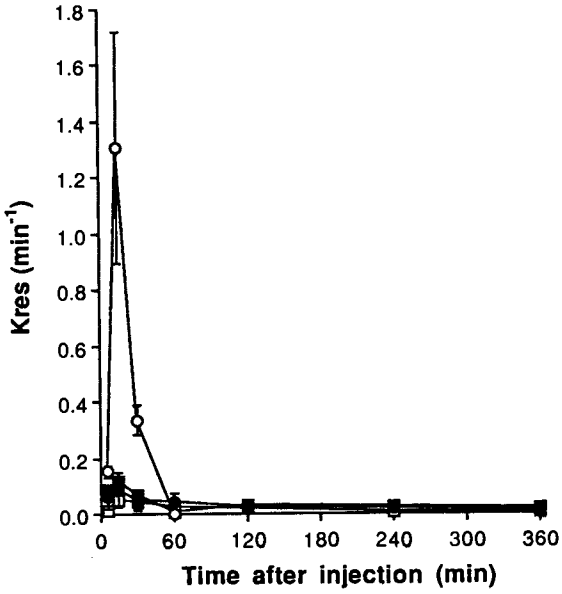

Fig. 5. k_{res}-time profiles after administration of liposomes. (■) is DPPC/SS (7:7)-liposomes, (□) is DPPC/SS (7:4)-liposomes, (●) is DPPC/SS (7:2)-liposomes, (○) is DPPC/SS (7:0)-liposomes. Values are mean ± SD (n = 3).

suggest that the liposomes of DPPC/SS (7:0) were easily taken up by the RES, the liposomes of DPPC/SS (7:2, 7:4, 7:7) have an ability to evade the uptake by RES and among them, the ability was the highest when the molar ratio of DPPC and SS was 7:4.

Comparison Between the Rates of Systemic Degradation and Uptake by RES

From Figs. 4 and 5, we found that the k_{res} is higher than the corresponding k_{deg} of liposomes after administration ($k_{res} > k_{deg}$). This result suggests that the uptake by RES is more important than the systemic degradation for liposomes with SS after administration since the RES recognizes the liposomes as "foreign bodies" and removes them from the blood. The high AUC value of the liposomes of DPPC/SS (7:4) may be mainly due to the lower k_{res} compared to the other liposomes (Fig. 5).

Comparing the Rate of Systemic Degradation with that of the Leakage of Calcein from Liposomes *in vitro*

The leakage of calcein from liposomes *in vitro* was studied by incubating 50 μl liposome suspension for 1 hour and 17 hours in 1 ml of 30% (v/v) rat plasma at 37 ± 0.5 °C, and the shaker speed was 100 min^{-1} (3). The leakage rate of calcein from liposomes (k_{leak}) is calculated from the slope of the percentage of the leakage of calcein from 0 to 17 hours except for the liposomes of DPPC/SS (7:0). The k_{leak} value in the liposomes of DPPC/SS (7:0) is calculated at an earlier time of from 0 to 1 hour since the liposomes of DPPC/SS (7:0) are unstable (3) and the liposomal calcein was eliminated from the blood 30 min after administration as described previously.

The results demonstrated that k_{leak} depended on the included amount of SS in the liposomes of DPPC/SS as summarized in Table I. The k_{leak} represents the average rate of calcein released from liposomes due to the collapse caused by the interaction of the bilayer of the liposomes with the proteins in plasma. We compared the k_{leak} *in vitro* with the k_{deg} for the liposomes of DPPC/SS (7:0, 7:2, 7:4, 7:7) after incubation and administration. Considering that the degree of leakage of calcein from liposomes is proportional to the plasma concentration (7, 8), the results indicated that the k_{deg} *in vivo* are almost the same as k_{leak} *in vitro* in spite of some error.

CONCLUSIONS

Measuring the levels of free calcein and liposomal calcein in the blood and applying clearance kinetics to these data, we evaluated the rates of systemic degradation (k_{deg}) and RES-uptake (k_{res}) for the liposomes of DPPC/SS after intravenous administration to mice. The results indicated that the rates of systemic degradation and the RES uptake of liposomes decrease with the addition of SS in DPPC liposomes. The rate of uptake by RES is larger than the rate of systemic degradation. The rate of leakage of calcein from liposomes by incubation in plasma *in vitro* was similar to that of the systemic degradation *in vivo*. The AUC of the liposomes of DPPC/SS (7:4) was dramatically higher than those having different DPPC and SS molar ratios possibly because of the lower rate of uptake by RES compared to other liposomes.

Table I. Comparison of the rate of leakage of calcein (k_{leak}) *in vitro* with the rate of systemic degradation (k_{deg}) *in vivo*

Liposomes composition DPPC/SS (molar ratio)	7:0*	7:2#	7:4#	7:7#
k_{leak} (hr^{-1})	23.1^a	0.600^c	0.233^c	0.455^c
k_{deg} (hr^{-1})	11.8^b	0.263^d	0.324^d	0.351^d

*: refer to the value at early times (a is the values from 0 to 1 hour (3), b is the values at 5 min in Fig. 4).
#: refer to the value at later times (c is the values from 0 to 17 hours (3), d is the average values from 120 min to 360 min in Fig. 4).

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