Formulation of Liposomes with a Soybean-Derived Sterylglucoside Mixture and Cholesterol for Liver Targeting

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In this study, we investigated a liposomal formulation of dipalmitoylphosphatidylcholine (DPPC) with a soybean-derived sterylglucoside mixture (SG) and cholesterol (Ch) for targeting the liver in mice. We found two distribution profiles of liposomes (reverse-phase evaporation vesicles, REV) in the liver depending on the concentration of Ch and SG *in vivo*; Ch tends to enhance liposomes containing 10 mol% SG accumulation in the liver, but to decrease those containing 20 mol% SG, and it prolongs the circulation in blood. Dicaptylphosphate did not enhance liver targeting by liposomes with SG and Ch. More DPPC/SG/Ch-liposomes (6:1:3) were significantly taken up by cultivated hepatocytes than DPPC/SG/Ch-liposomes (6:0:4), suggesting a glucose residue. The optimal composition for the maximal liver targeting was a mixture of 0.2 μ m DPPC/SG/Ch-liposomes (REV) at a molar ratio of 6:1:3. This composition of liposomes was distributed 3 times greater in hepatocytes than non-parenchymal cells 1 h after intravenous injection.

Key words liposome; sterylglucoside; hepatocyte; cholesterol; dipalmitoylphosphatidylcholine

Liposomes with conventional formulations are rapidly removed from blood circulation by the reticuloendothelial system (RES). Liposomes with greatly decreased uptake by the RES have been formulated, and their potential as drug carriers has been investigated in terms of targeting tissues, such as hepatocytes. Liposomes modified with glycolipids and glycoprotein carrying galactose residues are generally used to target hepatocytes.^{1,2)}

We reported that dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles (MLV) with a soybean-derived sterylglucoside mixture (SG) (DPPC/SG) are effective for targeting hepatocytes in mice, suggesting that SG at the surface of liposomes and their stability in the blood are important for liver targeting.³⁾ Therefore, we investigated formulations containing SG and cholesterol (Ch) for liver targeting, using reverse-phase evaporation vesicles (REV), which is superior to the entrapment efficiency of drug. Ch is used for long circulation in blood and targeting. First, to decrease uptake of RES, the ratio of Ch is important for the rigidity of the liposomes.⁴⁾ Fifty mol% Ch in liposome formulations containing polyethyleneglycol has been used to increase the circulation time and reduce uptake of RES.⁵⁾ Second, to target the liver, the functional group should project from the liposomes, which must be rigid and stable enough to encapsulate drugs. The ratio of Ch to phospholipid is

about 17 mol% for liposomes with asialoganglioside GM1 (ASG_{M1}) for liver targeting.⁶⁾ The optimal Ch ratio has not been clearly defined for liver targeting of liposomes.

We investigated liposomal formulations with SG and Ch for targeting hepatocytes. We examined the accumulation of various liposomes using calcein as a model drug in the liver *in vivo* and liposome uptake by cultured hepatocytes to investigate the function of the glucose group of SG.

MATERIALS AND METHODS

Materials DPPC, dicetylphosphate (DCP) and collagenase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calcein and Hank's balanced salt solution (HBSS) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Life Technologies, Inc. (NY, U.S.A.), respectively. Concanavalin A (Con A) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). SG was generously supplied by Ryukakusan Co., Ltd. (Tokyo, Japan). BCA protein assay reagent was purchased from Pierce (Rockford, IL, U.S.A.). SG is a mixture of steryl β -D-glucosides consisting of β -sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%) and brassicasterol (7.2%) (Fig. 1). All other chemicals were of reagent grade.

Fig. 1. Chemical Structure of SG

The numbers in parentheses represent the mixture ratio in SG.

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Male ddY mice weighing 27–35 g and Wistar rats weighing about 200 g were purchased from Saitama Experimental Animal Supply (Saitama, Japan).

Preparation of Liposomes MLV with entrapped calcein (20 mM in 1/10 diluted phosphate-buffered saline, 1/10 PBS, pH 7.31) were prepared with 70 μ mol DPPC and SG (DPPC:SG=7:2 and 7:7, molar ratio) by hydration, as described.⁷⁾ REV with entrapped calcein (20 mM in 1/10 PBS) were prepared from DPPC, SG, DCP or Ch (DPPC/SG/Ch or DPPC/SG/DCP/Ch) according to the REV method.⁸⁾ The liposomes were extruded through 0.1 or 0.2 μ m polycarbonate filters, then entrapped and free calcein were separated by gel chromatography. When liposomes are forced through filters with defined pore sizes, a liposomal population is obtained with a mean diameter that reflects the diameter of the filter pores.

Liver and Blood Distribution Mice were intravenously injected *via* the tail vein with a dose of liposomes entrapping calcein in 1/10 PBS (1.9–4.3 mg of total lipid/kg weight) or with free calcein in 1/10 PBS. Blood (10 ml) was sampled from the tail vein at specific times after the injection. The samples were immediately poured into 2.4 ml of PBS and mixed well, then centrifuged at $1000 \times g$ for 15 min. One hour later, the mice were sacrificed by cervical dislocation. The liver was removed and homogenized in 1/10 PBS. The total calcein concentration was expressed as the sum of the liposomal and released calcein from the liposomes. The liposomal calcein concentration was expressed as the fluorescence intensity of calcein in liposomes, calculated by subtracting the first fluorescence intensity of the sample from the total calcein fluorescence intensity after disruption by Triton X-100, as described previously.^{3,9)}

Fraction of Liver Cells The liposome suspension (4.7–6.0 mg of total lipids/kg) was intravenously injected *via* the tail vein of mice which were anesthetized with an i.p. injection of sodium pentobarbital (2 ml/kg). Liver perfusion was proceeded as described.⁷⁾ The liver was perfused *in situ* through the portal vein with Ca-free Hank's balanced salt solution containing 0.5 mmol/l glycoetherdiamine tetraacetic acid at 37 °C for 5 min, washed with 1/10 PBS for 5 min, then perfused with 0.05% collagenase for 5 min. The perfused liver was minced, separated into subfractions, and calcein was measured.

Aggregation of Liposomes with Con A The aggregation of liposomes with Con A was examined by measuring the turbidity of a liposome suspension at 360 nm after adding Con A at 25 °C.^{10,11)} The Con A concentration (0.097 μ mol/ml in 1/10 PBS) was equal to the amount of SG in the liposomes. The turbidity of the samples was corrected by subtracting the turbidity of a Con A solution.

Incubation with Hepatocytes in Primary Culture The liver was perfused with collagenase by a two-step procedure to obtain rat hepatocytes which were seeded at a density of 5×10^5 cells per 60 mm plastic culture dish coated with collagen.^{12,13)} The cells were incubated in Williams' E medium containing 10% fetal calf serum, 1.7×10^{-6} mol/l insulin, 10^{-5} mol/l dexamethasone and 100 units–100 μ g/ml penicillin–streptomycin mixture under a 5% CO₂ and 95% air atmosphere at 37 °C. After 24 h,

liposomes entrapping calcein were added to each dish. One hour later, unbound liposomes were removed by washing the cells 3 times with HBSS. Cells with bound liposomes were treated with a trypsin–EDTA mixture (1:4), then the fluorescence intensity was measured and the protein concentration of the cells was determined on representative dishes by a modified BCA protein assay.

Statistical Analysis Data from the experiments using mice were compared using analysis of variance and Student's *t*-test. A *p* value of 0.05, 0.01 or 0.001 was considered significant.

RESULTS AND DISCUSSION

Liposome distribution *in vivo* is a function of the size, membrane fluidity and liposome charge after intravenous administration. We initially compared REV with MLV composed of DPPC and SG for targeting hepatocytes. We then measured the % dose and the ratio of liposomal to released calcein in the liver as indicators of liposome uptake by the mouse liver to decide the optimal formulation of liposomes, in terms of size, SG and Ch concentration, and charged lipid (DCP). We also examined the distribution of the glucose group of SG by measuring liposomal uptake by hepatocytes in primary culture.

We reported that the ratio of the liposomes to the total calcein concentration in the blood of DPPC/SG-MLV (7:2, 7:7) was about 0.81, 1 h after intravenous administration, and that the uptake of DPPC/SG-MLV (7:2) in the liver was maximum at 20 min (about 80% of the dose), at which time the calcein level showed about 40% of the dose for 2 h. We also showed that DPPC/SG-MLV (7:2) was significantly taken up by hepatocytes, to a level about 7-fold greater than that by non-parenchymal cells.³⁾

The free calcein was rapidly eliminated from the blood: about 3% of the dose was in the blood 1 h after intravenous administration, and it did not accumulate in the liver (Fig. 2). The results suggested that if free calcein is released

Fig. 2 Time-Course of Blood Residence and Accumulation in the Liver after Intravenous Administration of Free Calcein

○, calcein in the blood; ■, calcein in the liver.

Table 1. Effect of Liposome Formulations and Size of MLV and REV with SG upon Liver Targeting

Composition (molar ratio)	Size ^{a)} (μ m)	Lip/Tot ^{b)} in liver	Blood/Liver ^{c)}	% Dose ^{d)} in liver	% Dose in blood
Free calcein	—	—	0.75 $\pm$ 0.26	2.8 $\pm$ 0.1	2.1 $\pm$ 0.7
MLV					
1. DPPC/SG (7:2)	0.1	0.35 $\pm$ 0.19	0.48 $\pm$ 0.19	46.4 $\pm$ 8.1	22.5 $\pm$ 7.8
2. DPPC/SG (7:7)	0.1	0.29 $\pm$ 0.32	0.43 $\pm$ 0.13	27.9 $\pm$ 3.5	12.0 $\pm$ 3.2
REV					
3. DPPC/SG (7:2)	0.1	0.55 $\pm$ 0.15	0.09 $\pm$ 0.01	63.7 $\pm$ 8.3	5.9 $\pm$ 0.5
4. DPPC/SG (7:7)	0.1	0.57 $\pm$ 0.15	0.11 $\pm$ 0.04	58.0 $\pm$ 9.5	6.5 $\pm$ 1.8
5. DPPC/SG/Ch (8:1:1)	0.1	0.16 $\pm$ 0.10	0.52 $\pm$ 0.07	23.2 $\pm$ 4.0	12.1 $\pm$ 3.0
6. DPPC/SG/Ch (7:1:2)	0.1	0.52 $\pm$ 0.07	0.52 $\pm$ 0.07	57.9 $\pm$ 3.2	19.2 $\pm$ 4.5
7. DPPC/SG/Ch (6:1:3)	0.1	0.53 $\pm$ 0.05	0.13 $\pm$ 0.11	65.8 $\pm$ 3.3	8.8 $\pm$ 7.5
8. DPPC/SG/Ch (5:1:4)	0.1	0.53 $\pm$ 0.25	0.12 $\pm$ 0.04	80.0 $\pm$ 17.3	9.3 $\pm$ 2.6
9. DPPC/SG/Ch (8:1:1)	0.2	0.61 $\pm$ 0.09	0.03 $\pm$ 0.01	51.4 $\pm$ 3.2	1.4 $\pm$ 0.4
10. DPPC/SG/Ch (7:1:2)	0.2	0.45 $\pm$ 0.10	0.13 $\pm$ 0.08	74.7 $\pm$ 6.5	6.4 $\pm$ 3.5
11. DPPC/SG/Ch (6:1:3)	0.2	0.67 $\pm$ 0.09	0.14 $\pm$ 0.03	80.6 $\pm$ 6.2	11.6 $\pm$ 1.9
12. DPPC/SG/Ch (5:1:4)	0.2	0.65 $\pm$ 0.22	0.07 $\pm$ 0.02	72.0 $\pm$ 16.0	5.2 $\pm$ 0.7
13. DPPC/SG/Ch (7:2:0)	0.1	0.55 $\pm$ 0.15	0.09 $\pm$ 0.01	63.7 $\pm$ 8.3	5.9 $\pm$ 0.5
14. DPPC/SG/Ch (7:2:1)	0.1	0.56 $\pm$ 0.17	0.25 $\pm$ 0.07	56.1 $\pm$ 9.2	14.1 $\pm$ 3.2
15. DPPC/SG/Ch (6:2:2)	0.1	0.53 $\pm$ 0.13	0.46 $\pm$ 0.25	50.8 $\pm$ 4.1	23.6 $\pm$ 12.3
16. DPPC/SG/Ch (7:2:1)	0.2	0.15 $\pm$ 0.09	0.54 $\pm$ 0.12	41.2 $\pm$ 5.0	22.2 $\pm$ 4.3
17. DPPC/SG/Ch (6:2:2)	0.2	0.41 $\pm$ 0.12	0.43 $\pm$ 0.06	45.6 $\pm$ 6.5	19.8 $\pm$ 0.4
18. DPPC/SG/Ch (5:2:3)	0.2	0.09 $\pm$ 0.24	1.90 $\pm$ 0.68	28.9 $\pm$ 6.9	54.9 $\pm$ 14.6
19. DPPC/SG/DCP/Ch (7:1:1:1)	0.1	0.31 $\pm$ 0.35	1.41 $\pm$ 0.65	26.1 $\pm$ 9.9	36.8 $\pm$ 9.4
20. DPPC/SG/DCP/Ch (6:1:1:2)	0.1	0	4.46 $\pm$ 2.03	18.4 $\pm$ 2.1	82.3 $\pm$ 36.2
21. DPPC/SG/DCP/Ch (5:1:1:3)	0.1	0	1.02 $\pm$ 0.08	14.2 $\pm$ 0.8	14.4 $\pm$ 0.8
22. DPPC/SG/DCP/Ch (7:1:1:1)	0.2	0.52 $\pm$ 0.05	0.11 $\pm$ 0.06	76.1 $\pm$ 4.8	8.2 $\pm$ 4.3
23. DPPC/SG/DCP/Ch (6:1:1:2)	0.2	0.43 $\pm$ 0.16	0.03 $\pm$ 0.01	74.2 $\pm$ 17.8	2.0 $\pm$ 0.5
24. DPPC/SG/DCP/Ch (5:1:1:3)	0.2	0.19 $\pm$ 0.10	0.66 $\pm$ 0.10	28.2 $\pm$ 0.5	18.6 $\pm$ 2.7
25. DPPC/SG/Ch (6:0:4)	0.2	0.06 $\pm$ 0.30	5.09 $\pm$ 0.53	15.1 $\pm$ 1.4	76.7 $\pm$ 3.7

a) The liposomes were extruded through 0.1 or 0.2 μ m pore size polycarbonate filters. b) Ratio of liposomal calcein in liver to total calcein in liver. c) Ratio of total calcein in blood to total calcein in liver. d) % dose of total calcein in liver. Liposome distribution in blood and liver were determined for calcein entrapped in liposomes 1 h after i.v. injection. Each data represents means $\pm$ S.D. ($n=3$). *t*-test in % dose in liver: 1, 4 $p<0.01$; 2, 4 $p<0.01$; 5, 6 $p<0.001$; 5, 7 $p<0.001$; 5, 8 $p<0.01$; 6, 7 $p<0.05$; 9, 11 $p<0.01$; 10, 11 $p<0.01$; 17, 18 $p<0.05$; 20, 21 $p<0.05$; 22, 24 $p<0.05$; 23, 24 $p<0.01$.

from liposomes in the blood, the free calcein dose not influence the accumulation of liposomes in the liver.

The accumulation of free calcein and DPPC/SG-liposomes (about 100 nm) in the liver and blood 1 h after intravenous administration in mice is shown in Table 1 (Nos. 1–4). The Lip/Tot liver value represents the ratio of the liposomal calcein to the liposomal and leaked calcein concentration (total calcein) in the liver. The Blood/Liver value is the ratio of the total calcein concentration in the blood to that in the liver. The ratio (%) dose in the liver value of DPPC/SG-REV (7:2 and 7:7) was about 60%, which was significantly greater than that of DPPC/SG-MLV (7:7). When liposomes are distributed into hepatocytes, the Lip/Tot liver values may be high, but they may be low when liposomes reach the RES, since liposomes are then broken. This value may indicate the distribution of liposomes in hepatocytes. This will be confirmed in the following hepatic cellular distribution.

The Lip/Tot value of REV was higher, whereas the Blood/Liver value was significantly lower than those of MLV (Nos. 1–4). This indicated that REV liposomal uptake by the liver was greater, and the % dose in the blood was lower than those of MLV.

Generally, the entrapment volume of REV is higher than that of MLV.^{13,14)} The entrapped volumes of DPPC/SG-MLV (7:2, 7:7) and DPPC/SG-REV (7:2, 7:7) were 1.26, 1.92, 2.31 and 4.78 (l/mol lipid), respectively. From these results, we can suggest that one

of the reasons for the larger entrapment volume of REV is that the liver was more efficiently targeted by REV than MLC.

Effect of Ch and Size of Liposomes Containing 10 mol% SG As the size of liposomes (REV) decreases, the uptake by the RES also decreases. Liposomes having an average diameter of approximately 100 nm were passed through the liver sinusoids and taken up by hepatocytes.¹⁵⁾ Therefore, we selected 0.1 and 0.2 μ m liposomes. When Ch is incorporated into liposomes, the membrane is more rigid and stable, and liposomes circulate longer in the blood with increasing Ch.¹⁶⁾ However, the optimal molar ratio of Ch is not clear for liver targeting.

We examined the effect of Ch and size of liposomes containing 10 mol% SG on the total calcein concentration in the liver and blood (Nos. 5–12). About 80% of the dose of liposomes containing 30 and 40 mol% Ch (0.2 μ m, size of liposomes, Nos. 11 and 12) and 40 mol% Ch (0.1 μ m, No. 8) was found in the liver. Both the Lip/Tot values in the liver and Blood/Liver values of liposomes containing 30 mol% of Ch (DPPC:SG:Ch = 6:1:3, 0.2 μ m, No. 11) were higher than those of other liposomes. The liver uptake of 0.1 (Nos. 5–8) and 0.2 μ m (Nos. 9–12) liposomes with 10 mol% SG increased with increasing Ch, but the % dose in the blood was almost constant. This suggested that Ch tends to enhance the accumulation in the liver of liposomes containing 10 mol% SG.

Allen *et al.*¹⁷⁾ reported that liposomes (REV) containing ASG_{M1} accumulated in RES about 70% of the dose, *i.e.*, about 0.46 in Blood/RES 2 h after intravenous administration. In this case, RES may almost express liver values, especially hepatocytes, because liposomes containing ASG_{M1} are well known to accumulate in the liver.⁶⁾ Therefore, the distribution ratio in the Blood/RES of liposomes containing ASG_{M1} was considered to be Blood/Liver. The % dose and Blood/Liver ratio in the liver of DPPC/SG/Ch-liposomes (6:1:3) were about 80.6% and 0.14, respectively. The % dose in the liver of DPPC/SG/Ch-liposomes (6:1:3) (80.6%) was similar to that of liposomes containing ASG_{M1} (70%), whereas the Blood/Liver value of DPPC/SG/Ch-liposomes (6:1:3) (0.14) was smaller than that of liposomes containing ASG_{M1} (0.46). This suggested that DPPC/SG/Ch-liposomes (6:1:3) were quickly removed from the blood circulation and taken up by the liver. The liver was targeted better by 0.2 than by 0.1 μm .

SG Concentration We reported that the amount of enzymatically detected SG on the MLV liposomal membrane is limited, and that the maximum molar ratio of SG in DPPC liposomes is about 0.27 (DPPC:SG = 7:2.6).¹⁸⁾ Therefore, we examined the effect of 20 mol% SG of liposomes on calcein concentration in the liver and the blood (Nos. 13–18). When the % dose in the liver decreased with increasing the Ch concentration in liposomes, the % dose in the blood increased (Nos. 13–15, 0.1 μm ; Nos. 16–18, 0.2 μm). This indicated that Ch was effective in stabilizing liposomes in the blood circulation, but did not improve liver uptake. The liver uptake of liposomes might depend on the fluidity of the liposomes since SG and Ch have opposite actions in stabilizing liposomes.⁸⁾ The % dose in the liver of 0.2 μm liposomes (Nos. 16–18) was lower and the % dose in the blood was higher compared with those in liposomes containing 10 mol% SG (Nos. 10–12). These indicated that Ch prolonged the circulation time more than the liver accumulation when liposomes contained 20 mol% SG (Nos. 13–18), in comparison to liposomes containing 10 mol% SG (Nos. 5–12).

We reported that liposomes need on optimally stable membrane for hepatocyte uptake.³⁾ Liposomes containing 10 mol% SG and Ch may provide optimal membrane stability for liver targeting compared with 20 mol% SG and Ch.

While the concentration of DPPC/SG/Ch-liposomes (6:0:4) in blood was very high, about 76% of dose, the accumulation of DPPC/SG/Ch-liposomes (6:0:4) in the liver was very low, about 15% of dose (No. 25). The Blood/Liver value of DPPC/SG/Ch-liposomes (6:0:4) was highest among these compositions of liposomes. Compared with DPPC/SG/Ch-liposomes (6:1:3) (No. 11), the accumulation of DPPC/SG/Ch-liposomes (6:0:4) in the liver was about 5 times lower, and concentration of DPPC/SG/Ch-liposomes (6:0:4) in blood was about 7 times greater than DPPC/SG/Ch-liposomes (6:1:3). This suggested DPPC/SG/Ch-liposomes (6:1:3) avoided the RES and SG and were effective in allowing liposomes to accumulate in the liver.

Effect of DCP In general, a negative surface charge

on liposomes protects them from degradation by a complement in the blood circulation.¹⁹⁾ We conferred a negative charge on the liposomal membrane by means of DCP.

We examined the effect of the DCP of liposomes containing SG on calcein concentration in the liver and blood (Nos. 19–24). About 20% of the dose of DPPC/SG/DCP/Ch-liposomes (0.1 μm) accumulated in the liver, whereas the Blood/Liver value was high (Nos. 19–21). More DPPC/SG/DCP/Ch-liposomes (0.2 μm) accumulated in the liver than those of 0.1 μm . The accumulation of liposomes in the liver decreased with an increase of Ch concentration to 10 mol% SG (Nos. 19–24), but the % dose in the blood increased with an increase of Ch concentration to 20 mol% SG (Nos. 13–18). These results indicated that Ch with DCP prolonged the circulation but did not improve liver accumulation, especially when liposomes were 0.1 μm .

DCP incorporated in 0.1 μm DPPC/SG/Ch-liposomes (Nos. 19–21) prolonged the circulation time, since the % dose in the blood was higher than DPPC/SG/Ch-liposomes (Nos. 5–8). In contrast, 0.2 μm DPPC/SG/Ch-liposomes with DCP (Nos. 22–24) did not tend to accumulate in the liver compared with those without DCP (Nos. 9–12), because the Lip/Tot value of liposomes with DCP (Nos. 22–24) was smaller than those without DCP (Nos. 9–12), and the value decreased with an increase of DCP. DCP may behave like SG in DPPC/SG/Ch-liposomes (20 mol% SG, Nos. 16–18), since the % dose in the liver decreased with an increase of Ch in DPPC/SG/DCP/Ch-liposomes (10 mol% SG, Nos. 22–24). DCP did not enhance liver targeting by liposomes with SG and Ch.

The Lip/Tot value and % dose in the liver values of DPPC/SG/Ch-liposomes (6:1:3, 0.2 μm) were the highest among the liposomes, and this composition provided optimal liver targeting.

Hepatic Cellular Distribution The hepatic cellular distribution of DPPC/SG/Ch-liposomes (6:1:3) and (6:0:4) 1 h after intravenous administration was examined to confirm the Lip/Tot liver values (Fig. 2). The size of each liposome was 0.1 and 0.2 μm . DPPC/SG/Ch-liposomes (6:1:3), both 0.1 and 0.2 μm , were more significantly taken up in hepatocytes than DPPC/SG/Ch-liposomes (6:0:4). The uptake of DPPC/SG/Ch-liposomes (6:1:3) of 0.1 μm in hepatocytes was to the same degree as that of 0.2 μm . When the uptake of DPPC/SG/Ch-liposomes (6:1:3) in hepatocytes was compared with that of DPPC/SG/Ch-liposomes (6:0:4), both sizes, 0.1 and 0.2 μm of DPPC/SG/Ch-liposomes (6:1:3), were taken up to a greater extent in hepatocytes than that of DPPC/SG/Ch-liposomes (6:0:4).

The proportion of hepatocytes and non-parenchymal cells in the liver is about 70 and 30%, respectively.²⁰⁾ We calculated that the ratio of liposomal calcein in hepatocytes to total calcein in hepatocytes of DPPC/SG/Ch-liposomes (6:1:3) of 0.1 and 0.2 μm was 0.47 and 0.48, respectively. These ratios corresponded well with the results of the Lip/Tot liver value, 0.53 (No. 7) and 0.67 (No. 11), in Table 1. This finding suggested that DPPC/SG/Ch-liposomes (6:1:3) in hepatocytes were taken up as unbroken liposomes.

Fig. 3. Hepatic Cellular Distribution of DPPC/SG/Ch-Liposomes (6:1:3 and 6:0:4, 0.1 and 0.2 μm) 1 h after Intravenous Administration in Mice
 ▨, total calcein in hepatocytes; □, liposomal calcein in hepatocytes; ■, total calcein in non-parenchymal cells; ▩, liposomal calcein in non-parenchymal cells.

Incubation with Hepatocytes in the Primary Culture

Figure 3 shows the aggregation of DPPC/SG/Ch-liposomes (6:1:3, 0.2 μm) induced by Con A in liposomes with or without SG. The turbidity of DPPC/SG/Ch-liposomes (6:1:3) increased, but that of DPPC/SG/Ch-liposomes (6:0:4) did not change for 120 min. Con A is a glycoprotein lectin that aggregates by recognizing both the galactose and glucose residues at the surface of liposomes.²¹⁾ Therefore, Con A can determine the presence of galactose and glucose. These results suggested that the glucose unit of SG was located at the surface of liposomes being recognized by Con A. We further examined the distribution of the glucose group of SG by measuring liposomal uptake by hepatocytes in primary culture.

Figure 4 shows the uptake after an incubation of DPPC/SG/Ch-liposomes (6:1:3, 0.2 μm) with hepatocytes for 1 h compared with liposomes without SG and exposed to Con A. First, we examined the influence of free calcein on uptake in hepatocytes for 1 h. About 17% of the free calcein was taken up by hepatocytes, compared with control DPPC/SG/Ch-liposomes (6:1:3). When both DPPC/SG/Ch-liposomes (6:1:3 and 6:0:4) entrapping calcein were incubated in Williams' E medium without hepatocytes for 1 h, the calcein retention by DPPC/SG/Ch-liposomes (6:1:3) (about 77%) was similar to that of DPPC/SG/Ch-liposomes (6:0:4) (about 80%). This suggested that the influence of free calcein released from DPPC/SG/Ch-liposomes (6:1:3 and 6:0:4) on the uptake in hepatocytes for 1 h was negligible. The uptake of DPPC/SG/Ch-liposomes (6:0:4) in hepatocytes was about 50% lower than that of DPPC/SG/Ch-liposomes (6:1:3). After incubation with Con A (1.67 $\mu\text{mol}/4\text{ml}/\text{dish}$) for 10 min at 37 $^{\circ}\text{C}$, the uptake of DPPC/SG/Ch-liposomes (6:1:3) decreased to the same level as that of DPPC/SG/Ch-liposomes (6:0:4). The glucose group of DPPC/SG/Ch-liposomes was masked by Con A without a change in liposome size (data not shown), therefore their uptake in hepatocytes decreased to the same level

Fig. 4. Aggregation of DPPC/SG/Ch-Liposomes (6:1:3, 0.2 μm) Induced by Con A

○, DPPC/SG/Ch-liposomes (6:1:3); ●, DPPC/SG/Ch-liposomes (6:0:4).

as that of liposomes without SG. The glucose group projecting outward from the liposomal membrane may contribute to the hepatic cellular distribution of liposomes. The uptake of DPPC/SG/Ch-liposomes (6:1:3) might be done in the liposomal state, not as free calcein from broken liposomes. The mechanism of hepatic uptake is still under investigation.

Our results show that the function of SG in targeting the liver was affected significantly by Ch. When Ch is added at the liquid-crystalline phase it stabilizes the liposomes, but at the gel phase it destabilizes them.¹⁶⁾ The stabilizing effect of Ch may increase blood circulation and partly improve liver targeting. REV may require rigidity of the liposomal membrane more than MLV because of their different numbers of bilayers. Therefore, the optimal composition of SG may be 10 mol% in REV with Ch, and 27 mol% in MLV without Ch.

Conclusions The accumulation of liposomes containing 10 mol% SG in the liver increased with an increasing concentration of Ch. Liposomes of 0.2 μm were more

Fig. 5. Uptake of Calcein after Incubation of DPPC/SG/Ch-Liposomes (6:1:3 and 6:0:4, 0.2 μ m) with Hepatocytes for 1 h at 37 °C

a) DPPC/SG/Ch-liposomes (6:1:3) were incubated after being pretreated with Con A for 10 min at 37 °C. Control is uptake of DPPC/SG/Ch-liposomes (6:1:3). *, $p < 0.01$ compared with control. Each value represents mean $\pm$ S.D. ($n = 3$).

effective for targeting the liver than those of 0.1 μ m. DPPC/SG/Ch-liposomes (6:1:3, 0.2 μ m) were accumulated in the liver to about 80% of the dose 1 h after intravenous injection and were taken up by hepatocytes in 3 times greater numbers than by non-parenchymal cells. DPPC/SG/Ch-liposomes (6:1:3) were more significantly taken up by cultivated hepatocytes than DPPC/SG/Ch-liposomes (6:0:4), suggesting a glucose function. These findings suggest that DPPC/SG/Ch-liposomes (6:1:3, 0.2 μ m) effectively target hepatocytes because the glucose group of SG projects outward from the liposomal membrane and the stability in the blood is optimized by 30 mol% Ch.

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