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Effects of glycophorin and ganglioside GM_3 on the blood circulation and tissue distribution of liposomes in rats

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Summary

The effects of glycophorin (GP) and ganglioside GM_3 (GM_3) on the blood circulation and tissue distribution of liposomes were investigated in rats. Liposomes composed of dipalmitoylphosphatidylcholine (DPPC)/cholesterol (Chol) or sphingomyelin (SM)/Chol, which contained additionally GP and/or GM_3, were prepared and the blood concentration and tissue (liver, kidney, lung and spleen) distribution after intravenous administration were compared with the control liposomes which contained neither GP nor GM_3. The blood concentration of DPPC liposomes containing GP and GM_3 (GP-GM_3-Lip[DPPC]) was significantly higher than those of the other DPPC liposomes for up to 6 h after administration, and about 3.3-fold higher than that of control liposomes (Con-Lip[DPPC]) at 6 h. The liver uptake of GP-GM_3-Lip[DPPC] at 6 h was significantly reduced compared with that of Con-Lip[DPPC], while the spleen uptake was increased significantly. Similarly, in the case of SM liposomes, the liposomes containing GP and GM_3 (GP-GM_3-Lip[SM]) prolonged the blood circulation time of liposomes and reduced their uptake by the liver, and the blood concentration was about 3.7-fold higher than that of control liposomes (Con-Lip[SM]) at 6 h. It was found that GP and GM_3 were useful and valuable for prolonging the blood circulation time of liposomes and reducing the uptake of liposomes by the liver.

Introduction

Liposomes have been extensively used as models of biological membrane and drug carriers (Gregoriadis, 1984; Ostro, 1987). With respect to therapeutic applications, it has been said that liposomes are useful carriers which deliver the encapsulated drugs to the reticuloendotherial system (RES) (Alving et al., 1978). It is well known that after intravenous administration colloidal particles such as liposomes and emulsions are readily removed from the blood circulation owing to the uptake by cells of the RES, for example, Kupffer cells of liver and macrophages of spleen. To control the fate of liposomes in the blood

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circulation is of great importance not only for the controlled release of drugs from liposomes, but also for the targeting of drugs to tissues other than the RES.

Factors affecting the clearance from the blood circulation and tissue distribution of liposomes have been discussed (Senior, 1987; Juliano, 1988): particle size, lipid composition, etc. For example, small unilamellar vesicles (SUV) tend to remain in the blood circulation rather than other larger vesicles (Allen and Everset, 1983; Senior et al., 1985), and incorporation of cholesterol (Chol) into lipid bilayer membrane generally enhances the stability of liposomes in serum and reduces the clearance rate from the blood circulation (Senior and Gregoriadis, 1982), however, these approaches appear to have limited applicability. On the other hand, the surface modification of liposomes has been proposed as an effective approach: in particular, the incorporation of carbohydrate residue into liposomal membrane. In the case of serum protein, the presence of terminal sialic acid residues, which are acidic carbohydrates, has been found to play a very important role in prolonging the circulation time of serum protein (Morell et al., 1968). Recently, several kinds of glycolipids or glycoproteins, for example, sialoglycoprotein of human erythrocytes (Utsumi et al., 1983), monosialoganglioside (GM_1) (Allen and Chonn, 1987), sialoglycopeptide derived from fetuin (Saito et al., 1988), glucuronic acid derivative (Namba et al., 1990), etc., have been used to prolong the blood circulation time of liposomes and reduce the uptake by the RES.

In this study, we used glycophorin (GP) and monosialoganglioside GM_3 (GM_3) as surface modifiers of liposomes. GP and GM_3 are the major sialoglycoprotein and sialoglycolipid of the erythrocytes (Marchesi et al., 1972), and attempted to ascertain whether the preparation of erythrocyte-mimetic liposomes was possible by the use of GP and GM_3.

Experimental

Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC), bovine brain sphingomyelin (SM), Chol, L-α-dipalmitoylphosphatidic acid (DPPA), and inulin (Mol. Wt 5000) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The purities of the lipids were checked by thin-layer chromatography (chloroform/methanol/water 70 : 30 : 5, v/v). Inulin was used as received without further purification. Glycophorin A (GP, Mol. Wt 55 000) from human erythrocyte membranes was a generous gift from Dr Hideo Utsumi (Showa University, Tokyo, Japan). GM_3 was purchased from Funakoshi Chemical Co. (Tokyo, Japan). [^{3}H]Inulin was purchased from New England Nuclear Research (Boston, MA). All other chemicals were commercial products of reagent grade.

Preparation of liposomes

The compositions of liposomes used in these experiments are listed in Tables 1 and 2. Multilamellar vesicles (MLV) were prepared by the conventional method introduced by Bangham et al. (1965). Namely, the lipids and/or GP were dissolved in chloroform/methanol/water (150 : 75 : 1 by vol.) (MacDonald and MacDonald, 1975) in a small round-bottomed flask. In order to make a thin lipid film, the solvent was then removed by blowing nitrogen gas into the flask which was warmed in a water bath. The residual solvent was removed in a desiccator under reduced pressure for 1 h. Phosphate-buffered saline (PBS, pH 7.4) containing 1 mM inulin and 1.48 MBq/ml of [^{3}H]inulin was added and the lipid film was hydrated. The flask was then agitated on a Vortex mixer for more than 5 min above the gel-liquid crystalline phase transition temperature (T_c) of the lipid materials. To attain a more homogeneous size distribution of the liposomes, the liposomal dispersion was extruded once above T_c through a polycarbonate membrane filter of 0.2 μm pore size as described by Olson et al. (1979). After extrusion, the liposomal dispersion was subjected to centrifugation twice at 150 000 $\times g$ each for 1 h to remove inulin and [^{3}H]inulin which had not been encapsulated into the liposomes. The pellet was resuspended using PBS. The concentration of DPPC or SM in the liposomes was determined by enzymatic assay using a phospholipid B-test Wako (Wako Pure Chemical Industries, Ltd, Osaka, Japan), and the liposomal

dispersion was diluted with PBS so that the final concentration of DPPC or SM could become the initial value.

Transmission electron microscopy (TEM)

For electron microscopic observation of liposomes containing GP and GM_3, a negative staining technique was used. A drop of liposomes was mixed with 2% phosphotungstic acid solution on a Cu-grid 300 mesh and dried. The micrographs of liposomes were recorded using a Hitachi transmission electron microscope (Type H-500).

Animal experiments

Male Sprague-Dawley strain rats in the weight range of 180–220 g were used in all experiments. Rats (3–7 per group) were anesthetized with pentobarbital (i.p.), fixed on a board and then injected in the hind limb vein with 4 μmol total lipids in 0.25 ml liposomal dispersion per 100 g body weight. Blood samples (approx. 120 μl) were obtained from the carotid artery at 0.25, 0.5, 2, 4 and 6 h after injection. Then rats were killed, and liver, kidney, lung and spleen were removed, rinsed in PBS, and weighed.

Determination of radioactivity

Radioactivity in blood and tissues after intraveneous injection of liposomes encapsulating [^{3}H]inulin as an aqueous maker was determined as follows. A 50 μl portion of blood was put on the filter paper ($n = 2$), and then dried overnight at room temperature. Next, the samples were prepared by the combustion method (Automatic Sample Combustion System, Aloka ASC-113, Tokyo, Japan) using a liquid scintillation cocktail (Aquasol-II, New England Nuclear Research, Boston, MA). The [^{3}H]inulin radioactivity of the blood samples was evaluated with a liquid scintillation counter (Aloka LSC-700, Tokyo, Japan). The whole blood volume was estimated to be 6.5% of the total body weight of the rat, and the concentration of [^{3}H]inulin in the blood was expressed as a percentage of the injected dose. A part (approx. 0.8 g) of the liver or the entire kidney, lung and spleen was homogenized with 5 ml of PBS. Then a fixed volume of tissue homogenate was transferred to a liquid scintillation vial, into which liquid scintillation cocktail was added, and the radioactivity was determined with a liquid scintillation counter. The tissue distribution was expressed as a percentage of the injected dose.

Statistical analysis

Values were expressed as means $\pm$ S.D. For the tests between multiple groups, Tukey's test was applied. A p value of 0.05 or less was judged to be significant.

Results and Discussion

Morphology and size of liposomes

Fig. 1 shows an electron micrograph of liposomes composed of DPPC, Chol, GP and GM_3 (GP-GM_3-Lip[DPPC], Table 1). The closed spherical vesicles can be clearly visualized, and the size distribution of these vesicles is observed to be less than 200 nm. Although the data are not shown, similar results were observed in other liposomes. These results mean that, under our experimental conditions, almost equal size distributions of liposomes were achieved.

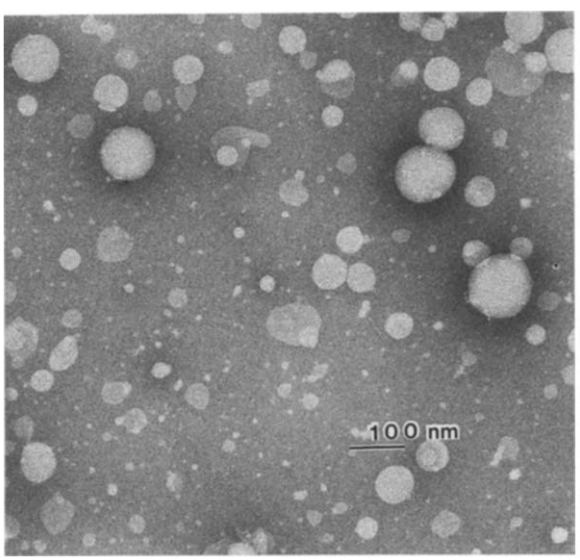

Fig. 1. Negative stain electron micrograph of liposomes composed of DPPC, Chol, GP and GM_3, passed through 200 nm pore size filter.

Effects of GP and GM_3 on the blood circulation and tissue distribution of DPPC liposomes

At first, the effect of GP and/or ganglioside GM_3, monosialoglycolipids, on the blood circulation and tissue distribution of DPPC liposomes was investigated. Table 1 lists the composition of the liposomes. The reason why [^{3}H]inulin was employed as an aqueous marker in liposomes is that free inulin which had leaked out from the liposomes is rapidly eliminated from the blood circulation and excreted to the urine without accumulation in the tissues.

Figs 2 and 3 depict the effects of GP and/or GM_3 on the blood clearance and tissue distribution of DPPC liposomes after intravenous administration. As shown in Fig. 2, the blood concentration of GP-Lip[DPPC] was significantly higher than that of Con-Lip[DPPC] at 2 h, but there were no significant differences between GP-Lip[DPPC] and Con-Lip[DPPC] at 4 and 6 h after administration, while the blood concentration of GM_3-Lip[DPPC] was no higher than that of Con-Lip[DPPC]. On the other hand, the blood concentration of GP-GM_3-Lip[DPPC] was significantly higher than those of the other DPPC liposomes for up to 6 h and about 3.3-fold higher than that of Con-Lip[DPPC] at 6 h. The liver uptake of GP-Lip[DPPC] and GP-GM_3-Lip[DPPC] at 6 h was significantly reduced compared with that of Con-Lip[DPPC], while spleen uptake was increased significantly. All liposomes in this experiment were distributed only to a very small extent to the kidney and the lung, and the incorporation of GP and/or GM_3 into the liposomal membrane did not affect the distribution to these tissues.

Durocher et al. (1975) suggested that the role of membrane sialic acid was important for erythrocyte survival. In the case of GP-Lip[DPPC], it is considered that the presence of sialic acid residues of GP on the liposomal surface plays an important role in the blood circulation of liposomes. On the other hand, Kojima and Hakomori (1989) have suggested that GM_3 liposomes interact with asialoganglioside GM_2 specifically, based on carbohydrate-carbohydrate interaction. This may indicate that GM_3-Lip[DPPC] would interact with any other glycolipids in vivo and GM_3 would not affect the blood concentration of liposomes, but further examination to clarify this point is necessary. In the case of GP-GM_3-Lip[DPPC], the blood concentration of the liposomes remained at a higher level compared with those of the other DPPC liposomes at all time points determined. These results suggested that the combination of GP and GM_3 had greater effect than GP or GM_3 alone. Sugar residues of glycoproteins and glycolipids which extend from the surface of the cell membrane form a layer known as the glycocalyx. It has been reported that an interaction occurs between the sugar residues of GP and ganglioside based on the hydrogen bonding of sugar residues (Sharom and Grant, 1978), which could affect the conformation of the sugar residues on the liposomal membrane (Endo et al., 1982). It can be postulated that the carbohydrate-carbohydrate interaction through hydrogen bonding between GP and GM_3 would affect the conformation of sugar residues of GP-GM_3-Lip[DPPC] and that the conformation would result in the high blood concentration of GP-GM_3-Lip[DPPC] and also the low uptake by the liver.

Effects of GP and GM_3 on the blood circulation and tissue distribution of SM liposomes

Next, the blood concentration and the tissue distribution of liposomes composed of SM, Chol

TABLE 1

Composition (μmol / ml) of liposomes

	DPPC	Chol	DPPA	GP	GM_3
Con-Lip[DPPC]	11.4	4.8	1.6	–	–
GP-Lip[DPPC]	11.4	4.8	–	7.3×10^{-3}	–
GM_3-Lip[DPPC]	11.4	4.8	1.6	–	7.2×10^{-2}
GP-GM_3-Lip[DPPC]	11.4	4.8	–	7.3×10^{-3}	7.2×10^{-2}

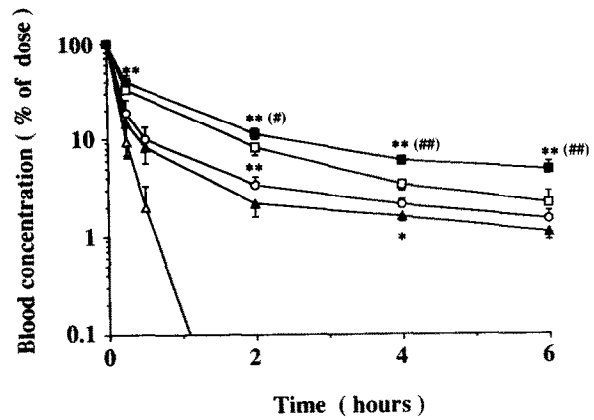

Fig. 2. Effects of GP and GM_3 on the blood circulation of DPPC liposomes (DPPC/Chol molar ratio, 7:3) Each bar represents the mean ± S.D. * $p < 0.05$, ** $p < 0.01$ compared with Con-Lip[DPPC]. # $p < 0.05$, ## $p < 0.01$ compared with GP-Lip[DPPC]. (○) Con-Lip[DPPC], (□) GP-Lip[DPPC], (▲) GM_3-Lip[DPPC], (■) GP-GM_3-Lip[DPPC], (△) inulin alone.

and DPPA in a molar ratio of 7:3:1 were determined. SM is believed to be a natural boundary phospholipid of the cell membrane (Huang, 1969) and hydrogen bonding among the amide groups of SM is considered to exist. For this reason, a mutual interaction with SM and GP and/or GM_3 in liposomal membrane was expected. The composition of SM liposomes used in this experiment is shown in Table 2.

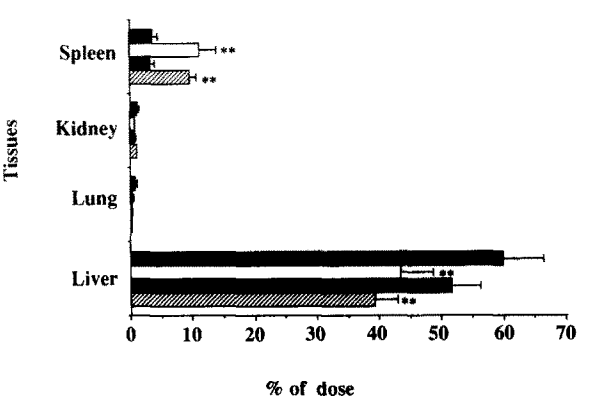

Fig. 3. Effects of GP and GM_3 on the tissue distribution of DPPC liposomes (DPPC/Chol molar ratio, 7:3) Each bar represents the mean ± S.D. ** $p < 0.01$ compared with Con-Lip[DPPC]. (■) Con-Lip[DPPC], (□) GP-Lip[DPPC], (⊠) GM_3-Lip[DPPC], (▨) GP-GM_3-Lip[DPPC].

TABLE 2

Composition (μmol / ml) of liposomes

	SM	Chol	DPPA	GP	GM_3
Con-Lip[SM]	11.4	4.8	1.6	–	–
GP-Lip[SM]	11.4	4.8	–	7.3×10^{-3}	–
GM_3-Lip[SM]	11.4	4.8	1.6	–	7.2×10^{-2}
GP-GM_3-Lip[SM]	11.4	4.8	–	7.3×10^{-3}	7.2×10^{-2}

Figs 4 and 5 show the effect of GP and/or GM_3 on the blood clearance and tissue distribution of SM liposomes after intravenous administration. As can be seen in Fig. 4, the blood concentration of GP-GM_3-Lip[SM] was significantly greater than those of the other liposomes at all time points determined and about 6.8-fold higher than that of Con-Lip[SM] at 2 h and 3.7-fold at 6 h. On the other hand, the blood concentrations of GP-Lip[SM] and GM_3-Lip[SM] were higher than that of Con-Lip[SM] at 2 and 4 h, while there were no significant differences among GP-Lip[SM], GM_3-Lip[SM] and Con-Lip[SM] at 6 h. As can be observed in Figs 2 and 4, the blood concentration of Con-Lip[DPPC] was higher than that of Con-Lip[SM]. The difference in blood concentration of these liposomes was considered to be dependent on the phase transition temperature of these phospholipids (DPPC, 41°C; SM, 32°C; Szoka and Papahadjopoulos,

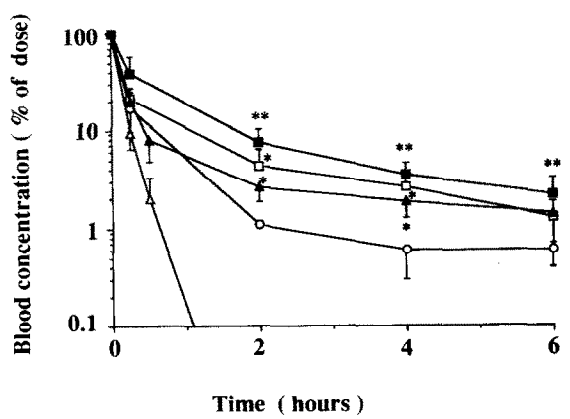

Fig. 4. Effects of GP and GM_3 on the blood circulation of SM liposomes (SM/Chol molar ratio, 7:3). Each bar represents the mean ± S.D. * $p < 0.05$, ** $p < 0.01$ compared with Con-Lip[SM]. (○) Con-Lip[SM], (□) GP-Lip[SM], (▲) GM_3-Lip[SM], (■) GP-GM_3-Lip[SM], (△) inulin alone.

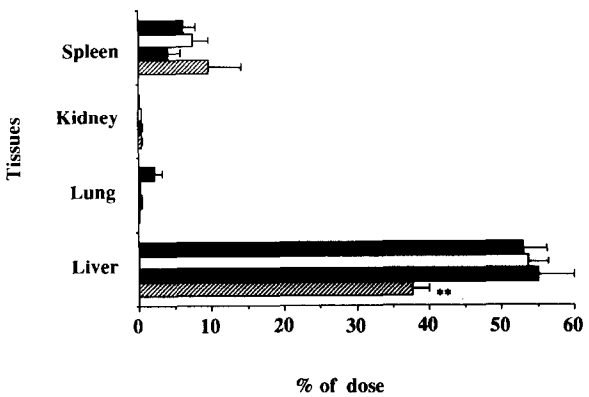

Fig. 5. Effects of GP and GM_3 on the tissue distribution of SM liposomes (SM/Chol molar ratio, 7:3) Each bar represents the mean ± S.D. ** $p < 0.01$ compared with Con-Lip[SM]. (■) Con-Lip[SM], (□) GP-Lip[SM], (▨) GM_3-Lip[SM], (▩) GP-GM_3-Lip[SM].

(1980)), and Con-Lip[DPPC] was considered more stable in the blood circulation. For the liver uptake of SM liposomes shown in Fig. 5, GP-GM_3-Lip[SM] at 6 h was only significantly reduced compared with the other SM liposomes. There were no significant differences in spleen uptake among these liposomes. It was evident from these results, that the combination of GP and GM_3 had a greater effect than GP or GM_3 alone, similarly to DPPC liposomes. Namely, it is also considered that the carbohydrate-carbohydrate interaction of GP and GM_3 through hydrogen bonding on the membrane of SM liposomes could affect the conformation of their sugar residues and that the conformation would give rise to the high blood concentration of GP-GM_3-Lip[SM]. In the case of GM_3-Lip[SM], its blood concentration was higher than that of Con-Lip[SM] at 2 and 4 h. This may suggest that factors such as the stability of GM_3-Lip[SM] in blood, the fluidity of the liposomal membrane, the interaction of the ceramide skelton between SM and GM_3, etc., are responsible, however, further examination to clarify the results is necessary.

Conclusion

Our results demonstrate that GP and GM_3 are useful and valuable for increasing the blood concentration and reducing the liver uptake of liposomes, as containing both GP and GM_3 reported here being considered as a new carrier. Liposomes containing GP and GM_3 are thought to mimic erythrocytes partly. It is believed that sialic acid can be useful not only for prolonging the blood circulation time of liposomes but also for their delivery to the desired organs. However, it is necessary to develop more simple and commercially viable derivatives instead of GP and GM_3 from the viewpoint of mass production.

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