Monosialoganglioside GM_1 Shortens the Blood Circulation Time of Liposomes in Rats

Dexi Liu,[1,2] Feng Liu,[1] and Young K. Song[1]

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Inclusion of monosialoganglioside GM_1 into liposomes significantly enhances the circulation time of liposomes in mice. Conversely, intravenously injected GM_1-containing liposomes were rapidly removed from the blood circulation and accumulated in the liver and spleen in rats. In rats, increasing the GM_1 content in liposomes resulted in more rapid clearance from the blood. Increasing the membrane rigidity of liposomes further increased the liver uptake of GM_1-containing liposomes. The activity of GM_1 in enhancing the liposome uptake by rat liver appears to be mediated by complement components.

KEY WORDS: drug delivery; ganglioside GM_1; liposomes; opsonin; reticuloendothelial system; complement system.

INTRODUCTION

Liposomes, being colloidal particles, are cleared from the circulation by the macrophages of the reticuloendothelial system (RES), mainly in the liver and spleen (1). Although this natural homing activity of liposomes has provided opportunities to deliver drugs to the RES, it has been an obstacle for the delivery of drugs to other cells (2). Attempts to reduce the affinity of liposomes to the cells of the RES and hence prolong their circulation time in blood has been one of the focuses of the liposome research in the last decade. Recently, liposomes that show prolonged circulation times in blood have been developed (3–9). The major characteristic of these long-circulating liposomes is the inclusion of ganglioside GM_1 (5,6), hydrogenated phosphatidylinositol (HPI) (6), or polyethyleneglycol conjugated to phosphatidylethanolamine (PEG-PE) (7–9) as part of the liposome composition.

Even though the elevated tumor accumulation and therapeutic advantages of such long circulating liposomes in cancer therapy have been demonstrated (4,6,10), the mechanism(s) leading to such prolonged circulatory half-life has not been fully elucidated. Earlier studies with cultured macrophages demonstrated the importance of serum opsonins such as complements, immunoglobulins and fibronectin in enhancing liposome uptake by macrophages (11–13). Results of recent studies showed that the blood half-lives of liposomes are inversely proportional to the total amount of blood proteins bound to these liposomes (14). In this respect, coating of liposomes with GM_1, PEG-PE or HPI may reduce the binding of serum components to liposome surface, resulting in a decreased liposome affinity for the RES and therefore prolonged circulation time. Alternatively, evidence has also been obtained suggesting that removal of liposomes from the blood is determined by the surface properties of liposomes. Liposomes of different lipid compositions and surface properties exhibit different affinities for interaction with the RES which then affects their circulation time in the blood (15). The reduced rate of liposome clearance by inclusion of GM_1, HPI or PEG-PE into the liposome bilayer may result from reduction of direct liposome affinity to the RES due to sterical stabilization of the liposomes (4). Furthermore, it has also been suggested that some particles such as liposomes and pathogenic microorganisms can attract suppressive substances called dysopsonins from the blood and thereby minimize their interaction with phagocytes (16,17). Thus, the prolonged circulation half-lives of liposomes may also be related to the binding of dysopsonins onto their surface.

In this report, we present the evidence that the function of GM_1 in prolonging liposome circulation time is animal species dependent. In mice, inclusion of GM_1 into liposomes decreases liposome affinity for the RES and results in a prolonged half-life in the blood. However, liposomes containing the same glycolipid showed reduced half-life in the blood and increased liver uptake in rats. We also demonstrate that in rats GM_1 in liposomes serves as a signal for RES clearance. The higher the GM_1 concentration in liposomes, the less time they stay in the blood. Employing liver perfusion techniques, we demonstrate that the accelerated removal of GM_1-containing liposomes from the blood of rats is likely mediated by serum components.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC), distearoylphosphatidylcholine (DSPC), bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids. Ganglioside GM_1 was from Supelco and cholesterol (Chol) was from Sigma. ^{111}In-diethlenetriamine pentaacetic acid distearylamine complex (^{111}In-DTPA-SA) was prepared as described previously (10). Animals used were from Harlan Sprague Dawley, Inc.

Liposome Preparation

Liposomes composed of PC/Chol/X (X represents the third lipid component) with a mole ratio of 10:5:1 were prepared by extrusion (10). Briefly, lipids with a trace amount of ^{111}In-DTPA-SA as lipid marker were dried under N_2 gas and vacuum desiccated for at least 30 min. The lipid film was hydrated in PBS (pH 8.0) overnight at room temperature. DSPC-based liposomes were hydrated at 65°C, 10°C above the T_m of DSPC. The lipid suspension was then extruded 20 times through two layers of polycarbonate filters (Nucleopore) with pore size of 100 nm using a LiposoFast extruder (Avestin Inc.). The average diameter of liposomes measured by a submicron particle analyzer (Colter N4SD) was 120 ± 25 nm.

Biodistribution Studies

Liposomes were injected via the tail vein into either

[1] Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania 15261.
[2] To whom correspondence should be addressed.

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mice (NIHS-3, male, 25–30 g) or rats (Sprague Dawley, male 300–350 g) anesthetized by ether at a dose of 5 μmole lipid/Kg in a volume of 100 μl for mouse or 200 μl for rat. At different time intervals after liposome injection, approximately 50 μl (mouse) or 200 μl (rat) blood samples were collected via the tail vein. The exact volume of blood in each sample was determined by weighing. The blood concentration of liposomes at the various time intervals was calculated based on the assumption that amount of blood is 7.4% of body weight for mice (18) and 5.4% for rats (19).

Liposome distribution in different organs was analysed 4 hour after administration. Mice were bled via the retro orbital sinus and killed by cervical dislocation. Blood and other organs were collected and weighed, and the radioactivity in each organ was counted in a gamma counter. Blood contamination into each organ was corrected by using correction factors as described previously (10). Liposome distribution studies in different organs for rats were performed in the same way, except that blood was collected via the portal vein to insure maximum removal.

Single-Pass Perfusion Studies of Liposome Uptake by Liver

GM_1-containing liposomes (1 μmole lipids) were incubated with either saline or 33% freshly collected serum in saline for 10 min at 37°C. The mixture was then perfused into the liver of an anesthetized rat via the portal vein at a rate of 7 ml per min. The inferior vena cava was cut at the beginning of the perfusion to drain the perfusion solution. The perfused liver was then washed with 20 ml of saline at the same perfusion rate. The amount of liposomes taken up by the liver was measured by counting the radioactivity in the liver.

RESULTS AND DISCUSSION

Comparison of Time-Dependent Clearance of GM_1-Containing Liposomes in Mice and Rats

In this study, the blood concentration of GM_1-containing liposomes as function of time was examined in mice and rats. Liposomes composed of PC/Chol/GM_1 (10:5:1, molar ratio) from a single batch were intravenously injected into animals at a dose of 5 μmole lipid/Kg. Blood samples from each animal were collected via the tail vein at 5, 15, 30, 60 and 120 min. As shown in Figure 1, the clearance rates of liposomes from the same batch were very different in mice and rats. The blood clearance of liposomes in mice was log-linear with a $t_{1/2}$ of approximate 6 hours while the elimination of liposomes from the blood of rats was much faster and showed non-linear, biphasic pharmacokinetics. Biphasic clearance kinetics is typical for conventional liposomes in the mice (20), indicating the involvement of two different elimination mechanisms. The effect of GM_1 in prolonging the circulation time of liposomes is not seen in rats. For example, at 2 hour, over 80% of the injected dose was found in the blood of mice compared to less than 2% in rats. Such rapid clearance of liposomes in rats is not function of anesthetization or the weight of the animals. Unanesthetized animals, with body weight ranging from 200 to 400 g gave similar results (data not shown).

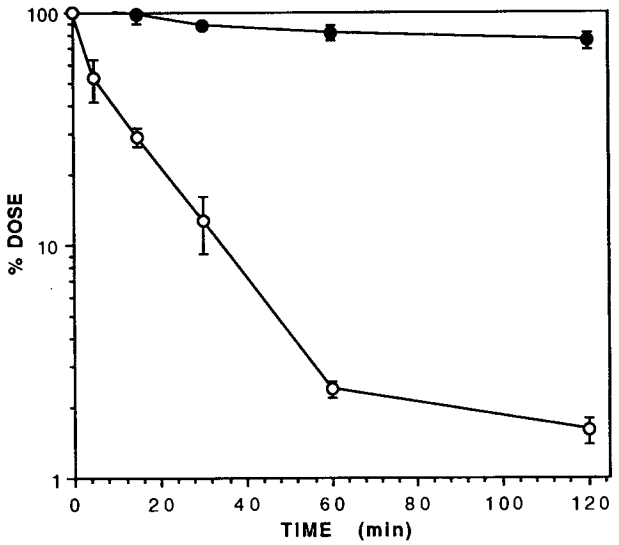

Fig. 1. Blood clearance of liposomes composed of PC/Chol/GM_1 (10:5:1, molar ratio) in mice (●) and rats (○). ^{111}In-labeled liposomes were injected intravenously at a dose of 5 μmole lipid/Kg. Blood samples were collected from animals at different intervals and the total amount of liposomes in the blood was calculated based on counting the radioactivity of each sample. Data are presented as mean ± S.D. (n = 3).

Organ Distribution of GM_1-Containing Liposomes

To determine the organ distribution of GM_1-containing liposomes in mice and rats, animals were sacrificed 4 hour after liposome injection, and the amount of liposomes in different organs were subsequently analyzed. About 60% of the injected liposomes was recovered from the blood of mice compared to less than 1% from the blood of rats (Fig. 2). In rats, liposomes accumulated mainly in the liver (60%) and spleen (22%) while other organs, including kidney, lung and heart showed minimum accumulation.

The species difference in the effects of GM_1 on liposome

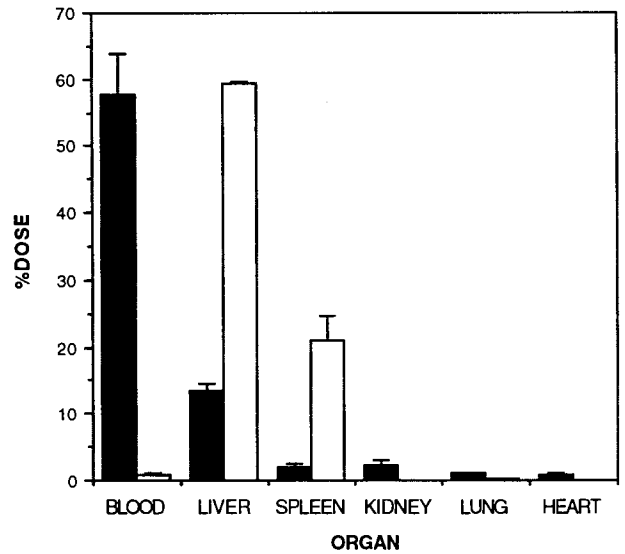

Fig. 2. Tissue distribution of PC/Chol/GM_1 (10:5:1) liposomes in mice (■) and rats (□) 4 hour after i.v. injection. Data were presented as mean ± S.D. of 3 animals for each species.

circulation time observed (Figs. 1 and 2) have led us to examine whether other lipid components would exhibit similar variations in mice and rats. Among the many lipids that have been investigated in the past, GM_1, PEG-PE, and PS were showed to have the most dramatic influence on liposome circulation time. In mice, both PEG-PE and GM_1, decrease the RES uptake of liposomes and increase the liposome half-life (3–9). In contrast, PS increases the RES uptake of liposomes and therefore decreases circulation time in blood (10,21). Since the activity of PEG-PE in prolonging the circulation time of liposomes in both rats and mice has been reported (for review, see ref. 3), we only tested the biodistribution of PS-containing liposomes in rats. As shown in Table 1, the corresponding activity of PS in regulating the biodistribution of liposomes in rats is the same as that in mice (10,21). PS-containing liposomes were rapidly cleared from the blood and accumulated in the liver and spleen. Over 80% of injected dose was found in liver and spleen at 4 hour while about 1% injected dose was found in the blood. The level of liposome accumulation in the RES for PS-containing liposomes is almost identical to that of GM_1-containing liposomes (84% in RES, 1% in blood). Therefore, GM_1 functions more like PS than PEG-PE in regulating the liposome circulation in rats.

To test whether the membrane rigidity of the GM_1-containing liposomes plays a role in determining the circulation time of liposomes in rats, we used DSPC as the matrix lipid for liposome preparation. Substitution of egg PC by DSPC enhanced liver uptake of GM_1-containing liposomes (Table 1). A more than 5-fold increase in RES/blood ratio was observed when DSPC instead of egg PC was used as the matrix lipid.

Effect of GM_1 Concentration in Liposomes on Liposome Blood Clearance

As shown in Table 1, GM_1 functions more like PS than PEG-PE in regulating liposome distribution in rats, i.e., GM_1 accelerates the removal of liposomes from the blood of rats. To confirm this conclusion, we examined the effect of GM_1 concentration in liposome membranes on blood clearance. Figure 3 is a semi-log plot of liposome concentration in blood as a function of time. An approximate 8-fold reduction of liposome concentration in the blood was seen at the 2 hour time point when only 1% GM_1 was included in the lipid mixture. The more GM_1 included in the liposomes, the faster they were cleared from the blood. For instance, 4 hour after administration, over an 100-fold decrease in the blood concentration was observed with liposomes containing 10% GM_1 as compared to liposomes without GM_1 (Table 1). The RES to blood ratios also increased with increasing amounts of GM_1 in the liposomes. The RES/blood ratio for liposomes containing 10% GM_1 was more than 300-fold greater than that of the liposomes without GM_1.

Table I. Biodistribution of Liposomes in Rats Four-Hour Post Injection

Liposome Composition (molar ratio)	% Injected Dose[a]			
	Blood	Liver	Spleen	RES/ Blood
PC/Chol/GM_1				
10:5:0	42.1 ± 0.6	21.2 ± 3.8	5.8 ± 0.5	0.6
10:5:0.15	2.9 ± 0.4	47.7 ± 5.4	26.4 ± 4.2	25.5
10:5:1[b]	1.0 ± 0.5	58.2 ± 3.1	26.8 ± 9.9	85.0
10:5:1.67	0.4 ± 0.1	71.3 ± 8.2	16.5 ± 5.2	219.5
DSPC/Chol/GM_1				
10:5:1	0.2 ± 0.0	70.0 ± 2.0	23.4 ± 0.9	467.0
PC/Chol/PS				
10:5:1	1.2 ± 1.3	73.1 ± 3.0	12.1 ± 2.4	71.0

[a] Data are given for blood, liver, and spleen which account for most of the dose injected. For other organs such as lung, heart, and kidney, % dose were below 1% and did not vary significantly among the liposome formulations tested. %injected dose in RES was calculated as the sum of liver and spleen. Data are presesnted as mean ± S.D. (n = 3–6).

[b] Data taken from Figure 2.

Single-Pass Perfusion Studies of Liposome Uptake by Rat Liver

Two possibilities can account for the action of GM_1 in rats. First, macrophages in liver and spleen of the rats may have a higher affinity for GM_1 and therefore liposomes are captured by the RES much easier when GM_1 is part of liposome composition, resulting in their rapid removal from the blood. Second, liposome uptake by the RES may be mediated by serum opsonin(s). To test these two possibilities, we have employed the single-pass liver perfusion system. Liposomes containing 6.25% GM_1 were incubated in saline with or without 33% serum in a volume of 20 ml for 10 min at 37°C. The mixture was then perfused through the portal vein into liver. Just before the start of perfusion the blood circulation was broken at the inferior vena cava to release the blood and later the perfusion solution from the liver. Liposomes were allowed to pass through the liver only once in

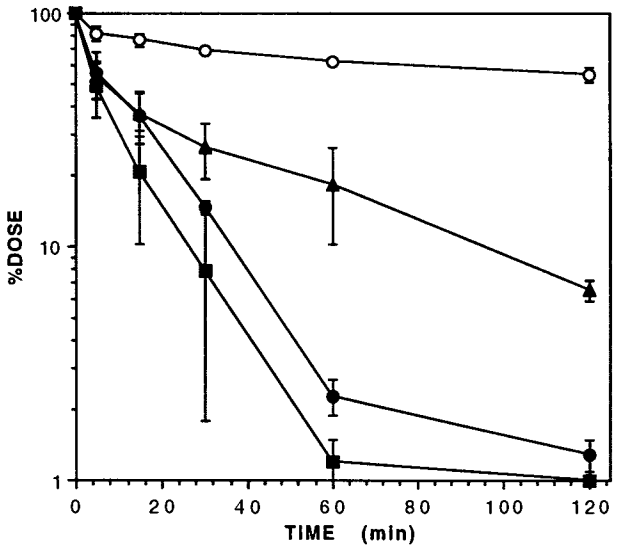

Fig. 3. Effect of GM_1 concentration in liposomes on blood clearance of liposomes in rats. ^{111}In-labeled liposomes containing the indicated amount of GM_1 were intravenously injected. The molar ratio of PC to Chol was kept at 10:5 for all liposome preparations. Blood samples were collected and analyzed as described in the method section. (○) PC/Chol without GM_1, (▲) with 1% GM_1, (●) with 6.25% GM_1, and (■) with 10% GM_1. Data are presented as mean ± S.D. (n = 3).

order to minimize potential damage to the liver during perfusion. As shown in Table 2, less than 5% of liposomes were found in the liver when liposomes were incubated with saline. However, 10% of liposomes were found in the liver after single passage when they were preincubated with rat serum. No increase in uptake was observed with liposomes preincubated with mouse serum. Rat serum did not show any effect on the total liposome uptake by the perfused liver for liposomes without GM_1 (Table 2). Heat treatment (56°C for 30 min) of rat serum to inactivate the complement system abolished the serum activity for GM_1-containing liposomes.

The results presented above clearly show that the activity of GM_1 in regulating liposome affinity for the RES and therefore blood circulation time is different in mice and in rats. GM_1 decreases liposome affinity for the RES and increases liposome circulation in mice, whereas this same glycolipid enhances the RES uptake and therefore decreases the circulation time of liposomes in rats. The differences in liposome uptake by the RES between mice and rats seems specific to GM_1 since the influence of both PS and PEG-PE on liposome circulation time are the same in both species (Table 1) (3,10,21).

While the evidence that GM_1 has opposite effect on liposome circulation time in mice and rats is clear, the explanation for the difference remains speculative. Elimination of serum activity by heat treatment (Table 2) suggests the involvement of complement components. This conclusion agrees well with the earlier reports that liposomes are recognized by complement components via the classical or alternative pathways, depending on the liposome composition (22–25). It is possible that GM_1-containing liposomes become coated with complement components when they are injected into rats and then are removed from the blood by the RES through complement receptors. Lack of activity in mouse serum (Table 2) would indicate that the complement system in mouse is not sensitive to GM_1. An alternative explanation is involvement of anti-GM_1 antibodies which exist in rats but not in mice. In this case, GM_1-containing liposomes are bound by anti-GM_1 antibodies in the blood and complement system will be subsequently activated. Liposome-bound complement components such as C3 may lead to the recognition of liposomes by the C3 receptors of the RES causing a rapid removal from the blood. These possibilities are currently under investigation in our laboratory and the results of these experiments will be presented in a separate report.

Table II. Effect of Serum on Liposome Uptake by the Liver in a Perfusion System[a]

Treatment	%Uptake[b]
PC/Chol/GM_1 (10:5:1)	
Saline	4.6 ± 0.2
Rat serum	10.0 ± 0.4
Mouse serum	5.0 ± 0.9
Preheated rat serum (56°C, 30 min)	3.4 ± 0.6
PC/Chol (10:5)	
Saline	2.2 ± 0.1
Rat serum	2.1 ± 0.1

[a] Liposomes (1 μmole total lipid) were preincubated with saline, normal or pretreated serum (33% in saline) for 10 min at 37°C. The mixture was then perfused through the liver as described under Materials and Methods.

[b] The total liver uptake was as % of total perfused liposomes. Data are presented as mean ± S.D. (n = 2–4).

While the present data clearly show the important role of serum factors in mediating the pharmacokinetic differences of GM_1-liposomes in rats and mice, it is evident that macrophages of the RES in rats may have a higher affinity for GM_1-containing liposomes than those in mice. As is shown in Table 2, liposomes without GM_1 only showed about 2% liver uptake independent of the presence of serum. This uptake represents only about half the amount of GM_1-containing liposomes taken up by perfused liver in the absence of serum. Therefore, the accelerated removal of liposomes containing-GM_1 from the blood of rats may involve serum factors in addition to the high affinity of liposomes for the RES.

It is premature to propose a definite mechanism for the activity of GM_1 described in this report. However, the results presented here support the conclusion that, in addition to various parameters in the liposome preparation, the long-circulating property of a given liposome composition in the blood may depend on the animal species. From the practical point of view, it will be important to examine the liposome circulation half life in a given animal species if the long circulating properties of liposomes are to be used.

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