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Serum independent liposome uptake by mouse liver

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Abstract

The rate of liposome clearance from blood by the reticuloendothelial system (RES), primarily the Kupffer cells of the liver, depends largely on liposome composition. Inclusion of phosphatidylserine or dicetyl phosphate into liposomes with a simple composition of phosphatidylcholine and cholesterol increases liposome clearance, while inclusion of GM_1 or amphipathic poly(ethylene glycol) decreases the rate of liposome clearance. To understand the underlying mechanism by which liposome clearance is regulated by the RES, we have developed a simple liver perfusion system. Using mouse liver as a model, we demonstrated that hepatic uptake of neutral or negatively charged liposomes does not involve serum components. Liver uptake of liposomes is directly related to the surface characteristics of liposomes. Liposomes with a neutral composition of phosphatidylcholine and cholesterol exhibit relatively low liver uptake. Inclusion of PS or DCP into these liposomes dramatically enhances liposome uptake by the perfused liver. Conversely, inclusion of GM_1 or PEG derivatives into liposomes greatly reduces the liposome uptake by the mouse liver. In contrast to the neutral or negatively charged liposomes, serum enhances the liver uptake of positively charged liposomes. Such serum effect on liver uptake of the positively charged liposomes is likely due to liposome aggregations caused by serum proteins. Inhibition of the liver uptake for PS-containing liposomes using liposomes with different compositions suggests that liver uptake of liposomes may involve different receptors.

Keywords: Liposome; Reticuloendothelial system; Liposome clearance; Drug delivery; Liver perfusion

1. Introduction

Much progress has been made in the last few years in the development of liposomes that have decreased affinity to the macrophages of the reticuloendothelial system (RES) and increased blood circulation half-life [1–4]. Such developments appear to have brought liposome research into a new era. Many reports have appeared in the literature demonstrating the increased therapeutic efficacy by using such long-circulating liposomes over those with conventional compositions [5–9]. While it is certain that more and more applications of such long circulating liposomes will be developed, the mechanisms by which these liposomes avoid the RES uptake are still unknown. A more general question still facing researchers is how the macrophages of the RES, primarily the Kupffer cells in the liver, differentiate between liposomes that have only subtle differences in their structure, including surface charge, size and lipid composition [3,10,24].

Analyzing the results of previous experiments that have used isolated cells as model system, two lines of evidence concerning this question have been accumulated. The hypothesis derived from the first line of evidence is called the opsonin dependent liposome clearance (for review, see [10]). It is hypothesized that liposomes, upon exposure to blood, become coated with plasma proteins. This process is thought to determine their recognition by the RES. Liposomes with different surface characteristics attract different arrays of plasma proteins. The interaction of these proteins with liposomes may also vary both in quantity and quality, depending on liposome surface structure. These differences may account for the different patterns in their blood clearance and tissue distribution [10,11]. Using primary culture of peritoneal macrophages as a model, early experiments have indicated that complement components [12], immunoglobulins [13] and fibronectin [14] may serve as opsonins to enhance liposome uptake. However, the

Abbreviations: Chol, cholesterol; DC-Chol, 3β-(*N*-(*N'*,*N'*-dimethyaminoethane)carbamoyl)cholesterol; DCP, dicetyl phosphate; DTPA-SA, diethylenetriamine pentaacetic acid distearylamine complex; GM_1, monosialoganglioside; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PEG-PE, poly(ethylene glycol) conjugated to dioleoylphosphatidylethanolamine; PS, phosphatidylserine; RES, reticuloendothelial system.

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activity of these components in the presence of other serum components have not been tested. It is not clear whether these components will have the same activity in the presence of high concentration of other serum proteins.

On the contrary, substantial evidence has also been accumulated in support of an opsonin independent mechanism [15,16]. This hypothesis emphasizes the importance of liposome-cell interaction. It is postulated that the clearance rate of liposomes from blood by the RES is determined by the surface characteristics of liposomes. Such characteristics can be directly recognized by receptors on macrophages of the RES and determine their affinity for the liposomes. Liposomes with higher affinity to these receptors are removed from the blood faster than those with low affinity. The recognition and uptake of liposomes by the RES does not involve the serum components [15,16]. Even though the results from such in vitro experiments correlated well with some of those from the in vivo experiments, one can not rule out the possibility that serum factors are involved in this process. This is because these results were obtained mainly in the absence or presence of fetal bovine serum specifically designed for cell culture and thus, may not contain all of the functional components that are involved in regulating liposome uptake by the macrophages.

Even though some of the in vivo results have been reproduced in these in vitro model systems, the major problem is that these results from these model systems were obtained under special conditions that may not represent the real situation in vivo. The reasons that these systems have not been able to provide conclusive evidence with regard to the mechanisms of liposome uptake by the RES are many. First of all, macrophages used in those experiments may have lost or changed their key properties with regard to their interaction with liposomes. Secondly, the assay conditions for liposome uptake in vitro is very different from an in vivo situation. Liposomes in blood with high flow rate in the body may interact with macrophages of the RES differently from those in culture where the interactions between liposomes and macrophages are more static. Lastly, the anatomic structure of the macrophages in RES has been lost in cell culture. Kupffer cells directly isolated from perfused liver using collagenase have suffered the same problems [17].

In order to overcome these problems, we have explored the possibility of using a liver perfusion system to study such an important question. Liver perfusion is a special technique commonly used for studies of liver functions [18–21]. Compared to the isolated macrophages, liver perfusion better represents the physiological conditions of the liver because damage to the liver macrophages can be minimized and the anatomic structure and blood flow dynamic of the liver can be preserved under the experimental conditions.

In this report, we have systematically studied the effect of liposome composition on and the function of serum components in RES uptake of liposomes using a mouse liver perfusion system. A strong correlation between liposome uptake by the liver in in vivo and in vitro perfusion systems has been observed. Our results suggest that the clearance of neutral or negatively charged liposomes by the RES in mice is an opsonin independent process and does not involve serum components.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC) from egg yolk and phosphatidylserine (PS) from bovine brain were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol (Chol) and dicetyl phosphate (DCP) were from Sigma (St. Louis, MO). Monosialoganglioside (GM_1) from bovine brain was purchased from Matreya (Pleasant Gap, PA). Diethylenetriaminepentaacetic acid stearylamide (DTPA-SA) [22], 3β-(*N*-(*N'*,N'-dimethylaminoethane)carbamoyl)cholesterol (DC-Chol) [23] and dioleoyl *N*-(monomethoxy poly(ethylene glycol) (M_r = 2000) succinyl)phosphatidylethanolamine (PEG-PE) [4] were kindly provided by Dr. Leaf Huang (Department of Pharmacology, University of Pittsburgh). $^{111}InCl_3$ (carrier-free) was from New England Nuclear (Wilmington, DE). Mice (NIH Swiss, male, 20–25 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN).

2.2. Preparation of liposomes

Large multilamellar liposomes were prepared at room temperature by the method of extrusion [4]. Briefly, lipids dissolved in Cl_3CH (for PC, PS, Chol and DC-Chol) or Cl_3CH/methanol (1:1) (for GM_1 and PEG-PE) were mixed at a desired molar ratio and dried under a stream of N_2 gas. The dried lipid films were then vacuum desiccated for at least 1 h to remove the residual organic solvent. A trace amount of ^{111}In-DTPA-SA (< 2 mol% of the total lipids) was included as lipid marker. The lipid films after vacuum desiccation were hydrated in PBS (pH 7.4) at a lipid concentration of 10 mM overnight and the lipid suspension was vortexed and extruded 15 times through two layers of Nucleopore membrane filter with a pore size of 0.8 μm using LiposoFast extruding device (Avestin, Ottawa, Canada). The average diameter of the resulting liposomes was about 350–450 nm as measured by dynamic light scattering using a Coulter N4SD particle size analyzer (Coulter Electronic, Hiateah, FL).

2.3. Single pass mouse liver perfusion

Liposomes (0.12 μmol total lipids in 12 μl) were mixed with 200 μl of either mouse serum (freshly collected) or buffer (Krebs-Henseleit buffer, pH 7.4) and

incubated for 10 min at 37°C. The mixture was then diluted to 2.4 ml with buffer and 2 ml of the diluted mixture was perfused via the portal vein through the mouse liver which was prewashed with 3 ml of buffer (prewarmed to 37°C). Mice were anesthetized by 2,2,2-tribromoethanol (0.6 ml, 20 mg/ml, i.p.) during the experiment. The inferior vena cava was cut at the beginning of the experiments to drain the blood, immediately followed by washing and subsequent perfusion with liposomes. Liposomes were only allowed to pass through the liver once. Unbound liposomes in the liver were removed by washing the perfused liver with 5 ml of buffer via the same route. The perfusion rate was kept constant at 2 ml/min. The amount of liposomes taken up by the liver was analyzed by measuring the ^{111}In radioactivity in the liver and presented as percentage of total amount of liposomes perfused.

2.4. Turbidity measurements

Same amount of liposomes (0.12 μmol total lipids in 12 μl) as used for the perfusion studies was incubated with 200 μl of either PBS or mouse serum for 10 min at 37°C. The incubation mixture was then diluted with PBS to a final volume of 0.6 ml. The turbidity of the diluted liposome solution was immediately measured in a spectrophotometer at a wavelength of 600 nm.

2.5. Infusion of liposomes in live animals

Liposomes (0.1 μmol total lipids in 100 μl) were infused into the anesthetized mice via the portal vein in 1 min. At the end of the infusion, the inferior vena cava was cut to drain the blood followed by perfusing the liver with 5 ml of prewarmed buffer to remove any unbound liposomes in the liver. The total liver uptake of liposomes was then analyzed by measuring the amount of ^{111}In radioactivity in the liver using a gamma-counter.

2.6. Inhibition of liver uptake of PS-containing liposomes by liposomes with different composition

The inhibition experiments were performed in two ways. Firstly, different amounts of liposomes containing PC/Chol, PC/Chol/DCP or PC/Chol/PS were mixed with PS-containing liposomes (^{111}In-labeled) and the mixture was then perfused through the liver under the conditions described above. Secondly, liposomes (2 μmol total lipids) with different compositions were first perfused through the liver, the perfused liver was washed with 3 ml of buffer and the testing liposomes (^{111}In-labeled PS-containing liposomes, 0.1 μmol total lipids) were then perfused through the same liver. The liver uptake of PS-containing liposomes (testing liposomes) was then analyzed accordingly.

3. Results

3.1. Composition dependent liposome uptake by liver

Liposome composition has been known to be the most important factor determining the rate of liposome clearance by the RES and therefore the blood circulation time in vivo [1–4]. For example, inclusion of PS, PA, DCP or positively charged lipids into the liposome bilayer increases liposome clearance by the RES [24] while inclusion of GM_1 or amphipathic PEG derivatives decreases the rate of liposome clearance by the RES [1–4]. The question that needs to be answered, however, is how liposomes are recognized by the RES. The main dispute concerning the mechanism of liposome recognition between opsonin-dependent and opsonin-independent hypotheses is whether serum components play an important role in liposome recognition. To test the serum effect, we have chosen a few representative liposome compositions for our experiments. These compositions have been demonstrated to have very different effects in regulating the blood half-lives of liposomes [2–4,24]. As shown in Fig. 1, about 9% of

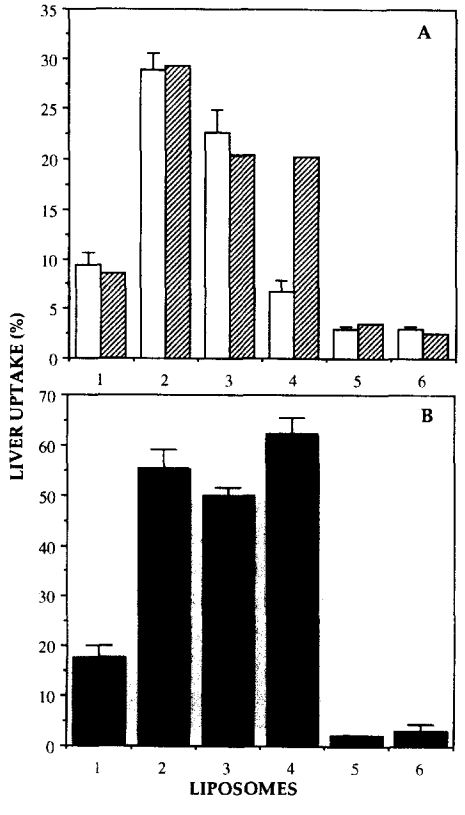

Fig. 1. Composition dependent liposome uptake by mouse liver. Liposomes (0.1 μmol total lipids) were either perfused (A) or infused (B) through the mouse liver according to the methods described in Section 2. 1, PC/Chol (10:5); 2, PC/Chol/DCP (10:5:1); 3, PC/Chol/PS(10:5:1); 4, PC/Chol/DC-Chol (10:5:1); 5, PC/Chol/GM_1 (10:5:1) and 6, PC/Chol/PEG2000-PE (10:5:1). (open bar) in buffer; (hatched bar) in serum ($n = 3$).

the total perfused liposomes with a basic composition of PC/Chol (10:5, molar ratio) were taken up by the liver after a single pass perfusion. Inclusion of 6.25% (mol) PS or DCP into such liposomes increased the total liver uptake to 23 and 30% respectively. By contrast, liposomes containing equal mol% of GM_1 or PEG-PE (PC/Chol/GM_1 or PEG2000-PE = 10:5:1) decreased the total liposome uptake by the liver to less than 3% (Fig. 1A). Under such conditions, preincubation of liposomes with freshly collected mouse serum (200 μl) did not affect the total liposome uptake. Using plasma (200 μl), blood (400 μl) or larger amount of serum (400 μl) did not change the total liposome uptake by the liver (data not shown). Interestingly, the only case where serum played a significant role in affecting the total liver uptake of liposome under such conditions was when liposomes contained 6.25% DC-Chol. The liver uptake of liposomes containing DC-Chol was enhanced about 3-fold in the presence of serum.

To demonstrate that the high liver uptake of liposomes in the absence of serum is truly serum independent and not due to the residual amount of unwashed blood components in the liver, we have varied the volume of washing buffer and looked whether the total liposome uptake by the perfused liver is affected by the volume of washing buffer used. The logic of this approach is straight forward, if the liver uptake of liposomes in the absence of serum is due to the small amount of unwashed blood components left in the liver, increase of the amount of washing buffer should decrease the amount of blood contaminant in the liver and, therefore, reduce the total liver uptake of liposomes. Using PS containing liposomes as an example, it was found that the liver uptake of PS liposomes by the perfused livers remained constant between 20–25% regardless of the volume (ranging from 3 to 10 ml per mouse liver) being used for washing prior to the administration of liposomes, suggesting that the liver uptake of those liposomes is truly through direct recognition.

To confirm whether the pattern of liposome uptake by the liver in our perfusion system correlates well with those in vivo, we infused the same amount of liposomes via the portal vein into live animals under anesthesia over a period of 1 min (the same period used for perfusion). After infusion, an incision was made at the inferior vena cava to drain the blood and subsequently the liver was washed with 5 ml of buffer via portal vein to remove unbound liposomes in the liver. It is obvious from Fig. 1B that the pattern of liver uptake of liposomes under such conditions is similar to that from the perfusion experiments. Liposomes containing PS, DCP or DC-Chol, which exhibit high liver uptake in perfusion, showed much higher accumulation in the infused liver. Liposomes of PC/Chol showed a moderate liver uptake. Liposomes containing GM_1 or PEG2000-PE showed minimal liver accumulation. There was a 2–3-fold increase in total liposome uptake by the infused liver over that by perfused liver for liposomes composed of PC/Chol, PC/Chol/PS, PC/Chol/DCP and PC/Chol/DC-Chol.

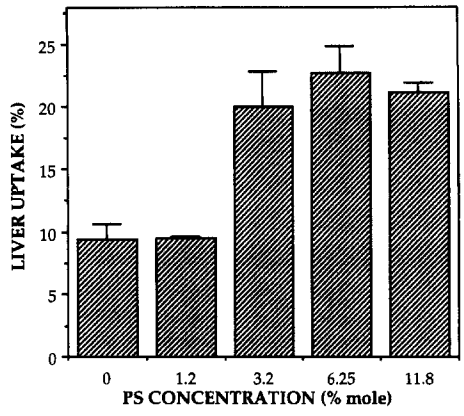

Fig. 2. PS concentration dependent liposome uptake by the perfused liver. Liposomes with various concentrations of PS were prepared using PC and Chol as matrix lipids (PC/Chol = 10:5, molar ratio). The concentrations of PS in liposomes are presented as mole percentage of the total lipids. The liver perfusions were performed in the absence of serum ($n = 3$).

3.2. *Effect of PS concentrations in liposomes on liver uptake*

The results shown in Fig. 1 strongly suggest that liver uptake of liposomes with a neutral or negatively charged surface is through direct recognition. Liposomes with a simple composition of PC/Chol showed a moderate amount of liver uptake (9%), suggesting a relatively low affinity for such liposomes by the Kupffer cells. Inclusion of PS or DCP into liposomes dramatically enhances the total liposome uptake by the perfused liver (23% and 30%, respectively), indicating that both PS and DCP in the liposomes serve as positive signal for liposome recognition by the Kupffer cells. If this is true, one would predict that the total liposome uptake by the liver should depend on the concentration of these components in liposomes. To test this hypothesis, we have prepared a series of liposomes by varying the concentration of PS in the bilayer and tested their liver uptake using our liver perfusion system. The results are summarized in Fig. 2. Liver uptake is very sensitive to the PS concentration in the liposomes. It required as little as 3% of PS in liposomes for the liver to reach maximum uptake. Further increases (up to 12%) of PS in liposomes did not result in any additional liposome uptake. Again, serum did not exhibit any effect on liver uptake of liposomes containing higher concentration of PS (data not shown).

3.3. *Effect of DC-Chol concentration in liposomes on the liver uptake of liposomes*

It is evident from Fig. 1 that serum enhances the liver uptake of liposomes containing DC-Chol. DC-Chol is a derivative of cholesterol that is commonly used as a

Table 1
Effect of the concentration of DC-Chol in liposomes on total liver uptake [a]

Liposomes PC/Chol/DC-Chol (molar ratio)	DC-Chol concentration (%)	Liver uptake (%)	
		buffer	serum
10:5:1	6.3	7.7±2.3	20.3±0.4
10:4:2	12.5	64.2±1.4	88.1±3.2
10:3:3	18.8	82.3±7.2	ND [b]
10:0:5	33.3	89.0±0.4	90.4±2.8

[a] Liposomes (0.12 μmol total lipids) with different concentration of DC-Chol in liposomes were incubated with either 200 μl of buffer or freshly collected mouse serum for 10 min at 37°C. The mixture was then diluted to 2.4 ml with prewarmed buffer and 2 ml of the diluted liposomes was perfused through the prewashed mouse liver. Data represents the average (S.D.) ($n \geq 3$)
[b] Not determined.

vehicle for gene delivery [23]. Theoretically, a high liver uptake of such positively charged liposomes should be expected considering the fact that the cell surface is negatively charged. The reason that only about 8% of liposomes (PC/Chol/DC-Chol = 10:5:1) were taken up by the perfused liver in the absence of serum is likely due to the insufficient concentration of DC-Chol in liposomes. Liposomes with 6.25% of DC-Chol may not show strong positive on their surface such that no strong electrostatic interaction between liposomes and liver cells will occur. To test this hypothesis, liposomes with increasing concentrations of DC-Chol were prepared and tested for their liver uptake in our perfusion system. As shown in Table 1, increase in DC-Chol concentration in liposomes resulted in an elevated total liposome uptake in the absence of serum. Liposomes containing 18% or greater of DC-Chol increased the total liver uptake of liposomes to over 80%. With 12.5% of DC-Chol, however, over 60% of the liposomes were taken up by the perfused liver. Again, serum enhances the liver uptake of these liposomes (Table 1).

3.4. Serum induced aggregation of liposomes containing DC-Chol

Given the fact that serum proteins are negatively charged under physiological conditions, we reasoned that the serum effect on the liver uptake of the positively charged liposomes may be due to the liposome aggregation induced by negatively charged serum proteins. To test such possibility, we measured the turbidity changes upon the incubation of liposomes with serum. As shown in Table 2, an increase in OD_{600} was observed after serum treatment for liposomes containing DC-Chol. The degree of turbidity increase depends on the DC-Chol concentration. Liposomes with 12.5% DC-Chol gave an OD_{600} of 0.122 compared to 0.097 when the concentration of DC-Chol was 6.3%. As a control, PS-containing liposomes showed no increase in turbidity. The OD_{600} for PS liposomes approximated to the sum of OD_{600} for liposomes (0.019) in PBS and diluted serum (0.054).

Table 2
Serum induced turbidity change of liposomes

Liposomes(molar ratio)	Size (S.D.) (nm)	Treatments	OD_{600} [a]
PC/Chol/DC-Chol (10:5:1)	432 (broad)	PBS	0.030
		serum	0.097
PC/Chol/DC-Chol (10:4:2)	392 (broad)	PBS	0.021
		serum	0.121
PC/Chol/PS (10:5:1)	355 (broad)	PBS	0.019
		serum	0.072

Liposomes (0.12 μmol total lipid in 12 μl) were incubated with 200 μl of either PBS (pH 7.4) or mouse serum for 10 min at 37°C and then diluted to a final volume of 0.6 ml with PBS. The turbidity of the samples was measured in a spectrophotometer at OD_{600}. The OD_{600} for PBS containing the same amount of serum without liposomes was 0.054.
[a] Data represent an average of duplicate experiments.

3.5. Liver uptake of PS-containing liposomes is mediated by specific receptors

It is evident from Fig. 1 that both PS and DCP-containing liposomes showed high liver uptake regardless of the presence of serum. One question we asked was whether such high liver uptake for both types of liposomes was mediated through the same receptors. To answer this question, a series of competition experiments was performed. In these experiments, ^{111}In-labeled PS-containing liposomes (testing liposomes) were mixed with increasing amounts of unlabeled liposomes of different compositions and then co-perfused through the liver. As shown in Fig. 3, the total liver uptake of testing liposomes was only sensitive to liposomes of the same composition (PC/Chol/PS = 10:5:1). The total liposome uptake of testing liposomes reduced from 20% in the absence of competing liposomes to about 5% when 2 μmol of liposomes of the same

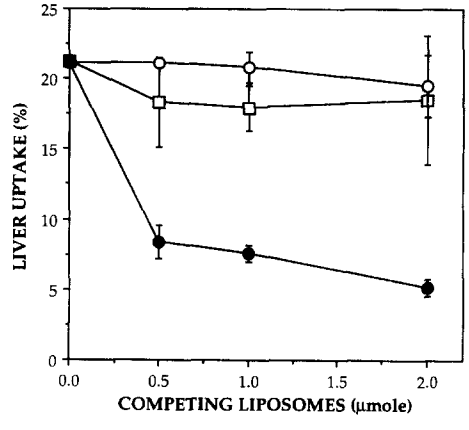

Fig. 3. Inhibition of the liver uptake of liposomes containing PS. ^{111}In-labeled PS-containing liposomes (0.1 μmol) were mixed with different amounts of liposomes composed of PC/Chol (10:5) (□), PC/Chol/DCP (10:5:1) (○) or PC/Chol/PS (10:5:1) (●) and the mixture was then perfused through the liver in the absence of serum ($n = 3$).

Table 3
Effect of pre-perfusion of liver by different liposomes on liver uptake of liposomes containing PS

Liposomes pre-perfused (molar ratio)	Liver uptake (%)
None	22.3 ± 1.2
PC/Chol (10:5)	21.2 ± 0.4
PC/Chol/DCP (10:5:1)	20.1 ± 1.6
PC/Chol/PS (10:5:1)	6.3 ± 0.5

The mouse liver was pre-perfused with either buffer or liposomes (2 μmol total lipids without radio label) of different compositions. The pre-perfused liver was then washed with 3 ml buffer and then perfused again with ^{111}In-labeled PS-containing liposomes (0.1 μmol total lipids). The methods of perfusion and the data analysis are the same as those in Table 1. Data represent average (S.D.) of three experiments.

composition were co-perfused. Interestingly, co-perfusion of the testing liposomes with liposomes either containing DCP or simply PC/Chol did not change the total level of liver uptake of the PS-containing liposomes (testing liposomes). For instance, 20-fold excess of liposomes with or without DCP over the testing liposomes did not affect the total liver uptake of the testing liposomes. Pre-perfusion of the liver with high dose of either PC/Chol or PC/Chol/DCP liposomes (2 μmol total lipids) did not decrease the total liver uptake of PS-containing liposomes either (Table 3), suggesting that the liver uptake of PS liposomes is through a specific mechanism that is different from those for DCP-containing or PC/Chol liposomes.

4. Discussion

We have clearly demonstrated in this report that the hepatic uptake of liposomes in mice does not seem to involve serum components (Fig. 1A). Liposomes with a basic composition of egg PC and cholesterol exhibit a basal level liver uptake, which can be modulated by the inclusion of additional membrane components. Inclusion of PS or DCP into the liposomes enhances the liposome uptake while inclusion of GM_1 or PEG2000-PE into liposomes reduces the total liposome uptake by the liver. The activity of the included lipids in modulating the liver uptake of liposomes depends on their concentration in the liposome bilayer (Fig. 2). The removal of PS-containing liposomes by the liver is likely mediated through membrane receptors that are different from those for DCP-containing liposomes (Fig. 3).

One exception to the above conclusion among the liposomes tested so far is liposomes containing positively charged lipids (PC/Chol/DC-Chol, Fig. 1 and Table 1). For positively charged liposomes, serum increases liver uptake. Such serum promoted liver uptake of liposomes, while seeming to support the notion that serum components play an important role in determining the affinity of the RES for the liposomes, is most likely through a nonspecific nature. It is likely that negatively charged serum proteins cause aggregation between positively charged liposomes (Table 2). The aggregated liposomes were then trapped in the liver while attempting to pass through the liver. An alternative explanation for the serum effect on these positively charged liposomes is that liposomes may be coated with serum proteins through electrostatic interaction between liposomes and serum proteins. The protein-coated liposomes were then taken up by the liver cells. The level of liver uptake of liposomes could be related to the amount of serum proteins bound per liposome which will be determined by serum to liposome ratio and charge density on liposome surface.

Comparing the total liver uptake of liposomes by the method of perfusion to that by infusion in live animals, the liver uptake of liposomes composed of PC/Chol, PC/Chol/DCP, PC/Chol/PS and PC/Chol/DC-Chol was 2- to 3-fold higher than those in perfusion (Fig. 1). This is likely due to the fact that liposomes infused into the live animals have multiple passes through the liver, while under our perfusion conditions, liposomes were only allowed to pass the liver once. For infusion, liposomes that escaped from the first pass through the liver may re-enter the liver and be taken up by the liver in the following pass. Nevertheless, the direct correlation between the uptake of liposomes by perfusion and infusion proves the usefulness and reliability of our in vitro liver perfusion system for studying the mechanism of liposome clearance by the RES.

While the evidence for direct recognition of liposomes by the liver cells is strong, the detail mechanism of such recognition is yet to be elucidated. For liposomes with no net charge (PC/Chol in our experiments) or with negatively charged surface such as liposomes containing PS or DCP, it has been well documented that Kupffer cells of the liver are directly involved [24]. In order for Kupffer cells to take up liposomes, liposomes have to bind to the surface of the Kupffer cells. The molecules that are responsible for such binding on the surface of the Kupffer cells should be able to differentiate the hydrophilic head groups of the liposomes. It has been postulated previously that scavenger receptors of the Kupffer cells are responsible for such recognition [27]. Obviously, our results in Fig. 3, although do not rule out the involvement of scavenger receptors, strongly suggest that other receptors are likely involved. The fact that the liver uptake of PS-containing liposomes can not be blocked by the DCP-containing liposomes, which also exhibit a high liver uptake both in vitro and in vivo, would suggest that these two types of liposomes are taken up by the Kupffer cells through different mechanisms. It will be interesting to know whether the uptake of other types of liposomes, including those containing PA and PG which show high rate of blood clearance, also involve different receptors.

In view of the liver uptake of positively charged liposomes in the absence of serum, we believe that the high level of liposome uptake by the liver is most likely due to the electrostatic interactions between positively charged

liposomes and negatively charged cell surfaces. Cells lining the blood vessels in the liver may be all involved. The involvement of hepatocytes is less likely concerning the diameter of the fenestration. The diameter (350–450 nm) of the liposomes used in our experiments is much larger than the pore size of the fenestration (about 100 nm in average [25]). The linear increase in liver uptake of liposomes with an increase in DC-Chol concentration in liposomes supports the notion of nonspecific binding.

One obvious question raised by our results is how to incorporate the results of previous experiments in the literature into the common scheme of the mechanism of liposome uptake by the RES. In fact, using rat liver perfusion systems, a few groups [19,20] including ours [21] have reported that serum opsonins are essential for liver uptake of liposomes. For example, Kiwada et al. [19,20] reported that liver uptake of liposomes containing DCP is solely dependent on the serum. We have also recently reported that uptake of PS-containing liposomes is also dependent on serum [21]. The obvious difference between those experiments and the experiments described in this report is the difference in the animal model. It is likely that different animal species use different mechanism in regulating the clearance of liposomes. In fact, such animal species dependent mechanism for liposome clearance has been previously reported [26]. We have recently found that liposomes containing GM_1 show an opposite effect in regulating liposome circulation time in mice and rats. In mice, it decreases the affinity of the RES for liposomes and prolongs the blood circulation half-life. While in rats it increases the affinity of the RES for the liposomes and, therefore, decreases their blood circulation half-life [26]. Such animal species dependent liposome uptake by the liver is also found in liposomes with other compositions (Liu, D. et al. [28]).

In summary, we have shown in an in vitro liver perfusion system that hepatic uptake of liposomes takes place through the direct recognition of the surface characteristics of liposomes by the Kupffer cells. No specific serum components are involved in such a recognition process for the liposomes tested so far. It appears that the rate of liposome clearance from the blood by the RES in mice, mainly the liver Kupffer cells, depends on the affinity of the Kupffer cells for the liposomes. Such affinity is determined by the liposome composition. Inclusion of PS or DCP into liposomes composed of PC/Chol increases the affinity and therefore enhances the liver uptake and reduces the blood circulation time. In contrast, inclusion of GM_1 or PEG-PE into liposomes of the same composition decreases the affinity, resulting in a prolonged liposome circulation time in blood. The function of GM_1 and PEG-PE in this case, however, is not to prevent the binding of the serum opsonins to liposome surface, instead, it is to sterically hinder the binding of liposomes to the receptor of Kupffer cells for PC/Chol liposomes. Characterization of the receptors that are responsible for the binding of liposomes and their relation to the binding of different liposomes are currently under investigation.

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