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Co-existence of serum-dependent and serum-independent mechanisms for liposome clearance and involvement of non-Kupffer cells in liposome uptake by mouse liver

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Abstract

The effect of serum on liver uptake of liposomes with different compositions was investigated using a single-pass liver perfusion technique. Among the liposomes tested are those containing CL, PA, DPGS, PE or glycolipids such as PI, GD, GT_{1b} and aGM_1. Liposomes containing PA, CL and DPGS showed high level of liver uptake in the absence of serum. Presence of serum decreased the total liver uptake for liposomes containing CL and PA by 50% and did not affect the level of liver uptake for DPGS-containing liposomes. The presence of serum, however, significantly increased the liposome uptake by the perfused liver for PG, PE and aGM_1 liposomes. Liposomes containing PI showed a minimal liver uptake regardless of serum presence. Fluorescence microscopic studies using a dual fluorescence label system in combination with Kupffer cell elimination technique showed that, in addition to the dominant role of Kupffer cells in taking up liposomes, non-Kupffer cells may also be involved in taking up CL and DCP-containing liposomes. Competition experiments using various liposome compositions indicated that liposome uptake by the liver cells may involve different receptors. Serum activity in enhancing the liver uptake for PE- and aGM_1-containing liposomes can be blocked by treatment of serum with EDTA, EGTA/Mg^{2+} and high temperature (56°C), suggesting the involvement of complement system. Results from this study support the conclusion that blood clearance of liposomes by the liver involves two independent mechanisms, one requires serum opsonins and the other does not.

Keywords: Liposome; Serum opsonin; Kupffer cell; Liposome clearance; Liver

1. Introduction

Liposomes have been considered as one of the most promising carriers for drug delivery [1–4]. Many reports have appeared in the literature in recent years, demonstrating that the therapeutic efficacy of a pharmaceutical agent can be significantly increased when administered in a liposomal formulation [1,2]. The functions of liposomes under such in vivo conditions include: (1) to protect the drug molecules against the degradation by body fluid, (2) to maintain a high drug concentration in the blood for a long period of time without causing serious toxic effect and (3) to concentrate the drug molecules at the target site through either an active or passive targeting mechanism. Another important factor that has become apparent from these in vivo studies is that one needs to control the blood kinetics and tissue distribution of the liposomes in order to further improve the therapeutic efficacy. For example, liposomes which can stay in circulation for prolonged period of time to extravasate the leaky vessels of solid tumor are very useful in delivering antitumor drugs for cancer chemotherapy [5–8]. To achieve this goal, however, we will have to understand the physical, chemical and biological factors that regulate the blood clearance and tissue distribution of liposomes.

It is known for some time that liposomes, once injected into the animals, are quickly eliminated from the blood by the reticuloendothelial system (RES), primarily the macrophages of the liver [9]. The clearance of liposomes

Abbreviations: aGM_1, gangliotetraosyl ceramide; Chol, cholesterol; CL, cardiolipin; Cl_2MBP, dichloromethylene bisphosphonate; DCP, dicetyl phosphate; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DPGS, 1,2-dipalmitoyl-*sn*-glycerol-3-succinate; DTPA-SA, diethylenetriaminepentaacetic acid stearylamide; GD_{1b}, disialoganglioside; GT_{1b}, trisialoganglioside; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

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from the blood is mainly dependent on liposome composition and diameter [10–12]. Generally, liposomes containing negatively charged lipids such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA) or cardiolipin (CL) are cleared much faster than those containing neutral lipids. However, inclusion into liposome bilayer of glycolipids such as monosialoganglioside (GM_1), phosphatidylinositol (PI) or amphipathic polyethylene glycol [13–15] significantly decreases the rate of liposome clearance. With respect to the effect of liposome size on the rate of blood clearance, liposomes with large diameters are cleared faster than those with smaller diameters of the same composition. However, the basic principles behind these well-known phenomena remain unknown. For example, it is not very clear why inclusion of small amount of specific lipid into the liposome bilayer could dramatically change the liposome retention time in the blood circulation and the level of liposome accumulation in the liver.

To understand the roles of these added lipids in affecting the liposome clearance by the liver, we have recently developed a single-pass liver perfusion model system [16,17]. Using mouse liver as a model, we have demonstrated that inclusion of negatively charged lipids such as PS and DCP into the liposomes increases the liposome uptake by the perfused liver. Inclusion of other types of lipids such as GM_1 or PEG-PE into liposomes decreases the liposome uptake by the perfused liver. Such liposome uptake pattern correlates very well with that obtained in vivo. Experiments designed to identify the factors that control the high liver uptake of liposomes containing PS and DCP showed that these types of negatively charged liposomes were taken up by the liver through a mechanism of direct recognition and without involving the serum opsonins [17]. As these results were obtained from a limited number of liposome compositions, we decided to test whether such serum-independent mechanism can apply to other types of liposomes. In this study, we have examined the effect of serum on the liver uptake of PC/Chol based liposomes containing CL, PA, PG, DPGS, PE, or glycolipids including PI, aGM_1, GD_{1b} or GT_{1b} using the same single-pass liver perfusion system. We also examined whether Kupffer cells are solely responsible for liver uptake of liposomes.

The results from these experiments as presented in this paper suggest that both serum-dependent and serum-independent mechanisms for liposome clearance coexist in the mouse. Depending on lipid composition, liposomes can be taken up by liver cells either through a serum-dependent mechanism or a serum-independent mechanism. We also demonstrated that while the Kupffer cells are the major cell type responsible for liposome uptake, it is evident that non-Kupffer cells in the liver may also participate in taking up liposomes with a particular composition. Results from experiments designed to characterize the molecules involved in liver uptake of different types of liposomes suggest the existence of multiple receptors for lipids.

2. Materials and methods

2.1. Materials

Phospholipids including egg phosphatidylcholine (PC) and phosphatidylethanolamine (PE), bovine brain phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), 1,2-dipalmitoyl-*sn*-glycerol-3-succinate (DPGS) and cardiolipin (CL) were from Avanti Polar Lipids (Birmingham, AL). Glycolipids including phosphatidylinositol, disialoganglioside (GD_{1b}), trisialoganglioside (GT_{1b}) and gangliotetraosyl ceramide (aGM_1) were purchased from Matreya (Pleasant Gap, PA). Cholesterol (Chol) and dicetyl phosphate (DCP) were ordered from Sigma (St. Louis, MO). Dichloromethylene bisphosphonate (Cl_2MBP) was a kind gift from Boehringer Mannheim, Germany. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was from Molecular Probes (Eugene, OR). The fluorescence beads (Fluoresbrite YG Microspheres, 1 μm in diameter) were ordered from Polyscience (Warrington, PA). $^{111}InCl_3$ (carrier free) was purchased from New England Nuclear (Wilmington, DE). Diethylenetriaminepentaacitic acid stearylamide (DTPA-SA) was kindly provided by Dr. Leaf Huang (Department of Pharmacology, University of Pittsburgh). Synthesis and ^{111}In labeling of DTPA-SA has been described previously [18]. Animals (CD-1 mice, male, 20–25 g) were from Charles River (Wilmington, MA).

2.2. Liposome preparation

Liposomes composed of PC/Chol and an additional lipid component with a molar ratio of 10:5:1 (PC/Chol/additional lipid)) were prepared by a method of extrusion [19]. For a standard preparation, lipids in the desired ratio were mixed in chloroform. The organic solvent was evaporated under a stream of N_2 gas and the lipid films were then vacuum desiccated for at least 2 h to remove the residual organic solvent. The dried lipid films were hydrated in PBS (pH 7.4) for a minimum of 2 h at a lipid concentration of 10 mM. The lipid suspension was then extruded 10 times through polycarbonate filter (Nucleopore) with a defined pore size using LiposoFast extrusion device (Avestin, Canada). Multilamellar liposomes prepared with an average diameter between 300–500 nm, as measured by light scattering using Coulter N4SD particle size analyzer (Coulter Electronic, Hiateah, FL), were used in this study.

To label liposomes, a trace amount of either ^{111}In-DTPA-SA or DiI (25 μg per 5 μmol lipid) were mixed with other lipids at the beginning of the liposome preparation.

Cl_2MBP-containing liposomes were prepared according to the method previously described by Van Rooijen [20]. Briefly, the dried lipid films containing 30 μmol total lipids (PC/Chol/PS = 10:5:1, molar ratio) were hydrated

in Cl_2MBP (75 mg/ml in H_2O). The osmolarity of the solution is equivalent to that of serum. Trace amount of [^{3}H]glucose was included as the water-soluble marker to estimate the entrapment efficiency of Cl_2MBP. The lipid suspension was then gently vortexed. To remove unencapsulated Cl_2MBP, the lipid suspension was first centrifuged for 30 min in a desk top centrifuge (Sorval RT600B) at a speed of 3000 rpm. Liposomes floated to the top under such conditions were collected and resuspended in 1.5 ml PBS (pH 7.4). The liposome suspension was then centrifuged in a microcentrifuge (12000 × g, 10 min). The liposome pellet was washed two more times under the identical procedure using the microcentrifuge and finally resuspended in 1 ml of PBS. The estimated encapsulation efficiency for Cl_2MBP under these conditions is about 30 μg per μmol total lipids.

2.3. Single-pass liver perfusion

Liposomes (12 μl containing 0.12 μmol total lipids) were mixed with 200 μl of either freshly collected mouse serum or PBS and incubated at 37°C for 10 min. The mixture was then diluted into 2.4 ml with prewarmed (37°C) Krebs–Henseleit Buffer (KHB) (pH 7.2, saturated with 95% O_2/5% CO_2). 2 ml of the mixture were then perfused through the prewashed (by 5 ml of prewarmed KHB, pH 7.2) liver via the portal vein. Animals were anesthetized by 2′,2′,2′-tribromoethanol (0.6 ml/mouse, 20 mg/ml, i.p.) for cannulation. An incision at inferior vena cava was made to drain the blood and the perfusate. Unbound liposomes in the liver were removed by perfusing through the liver with 5 ml of saline. The rate of perfusion was kept at 2 ml/min. The amount of liposomes taken up by the liver was analyzed by measuring the total ^{111}In radioactivity in the liver using a gamma counter and presented as the percentage of the total liposomes perfused.

For competition experiments, the test liposomes (^{111}In-labeled) were mixed with competing liposomes (2 μmol total lipids, without labeling) and the liver perfusion was performed using the protocols described above.

2.4. Serum treatment

To 200 μl of freshly collected mouse serum, either 24 μl of EDTA (100 mM) or 30 μl of EGTA/Mg^{2+} (80 mM/20 mM) was added. The final volume of the mixture was adjusted to 240 μl by saline. For control group, 40 μl of saline was directly added to the serum. The mixture was incubated for 30 min at 37°C before mixing with liposomes. Inactivation of complement components was performed at 56°C for 30 min using same amount of serum diluted with 40 μl saline.

2.5. Kupffer cell elimination

To each mouse, 100 μl of liposomes containing 3 μmol total lipids with or without Cl_2MBP entrapped inside were intravenously injected. 24 h after the injection, a single-pass perfusion was performed on these animals to examine the effect of the liposomal Cl_2MBP treatment on liposome uptake by the liver.

2.6. Fluorescence microscopic studies

Fluorescence-labeled polystyrene beads containing 1.2 · 10^9 particles in 100 μl were injected into the mouse via tail vein. 1 h post injection, animals were anesthetized and the liver perfusion was performed with DiI-labeled liposomes (2 μmol total lipids). Liver sections (10 μm in thickness) were immediately prepared by a standard cryosection procedure [21], observed and the pictures were taken under a fluorescence microscope.

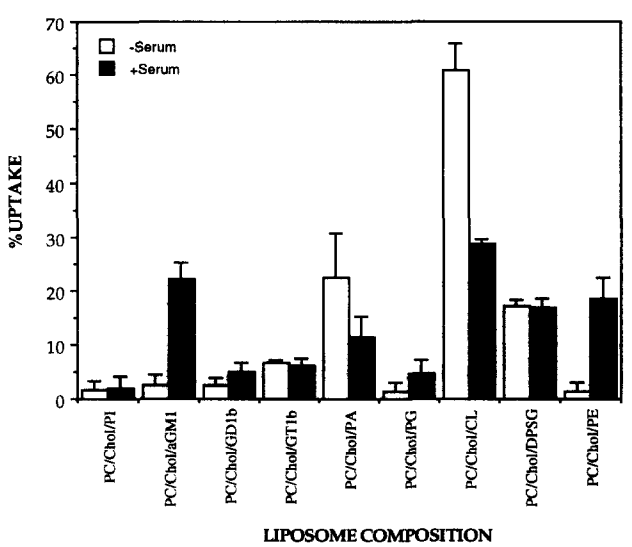

Fig. 1. Effect of serum on liposome uptake by the perfused mouse liver. Data represent mean ± S.D., n = 3–5.

3. Results

3.1. Effect of serum on liposome uptake

Fig. 1 shows the liver uptake of radio-labeled liposomes after single pass perfusion through the mouse liver for a variety of compositions, all with PC/Chol as the matrix lipid, and an average diameter of about 300–500 nm (mean from unimodel analysis). On the basis of the total liposome uptake by the perfused liver, three patterns were observed with respect to the effect of serum on liposome uptake by the liver: positive, negative and no effect. Serum enhanced the liver uptake of liposomes containing aGM_1, PG and PE. In the absence of serum, only about 2% of these liposomes were taken up by the liver after perfusion. The total liposome uptake in the presence of serum in-

creased to about 20% for aGM_1 and PE liposomes, and about 5% for liposomes containing PG. Under these experimental conditions, serum increased the total liver uptake of these liposomes by 3–10-fold, indicating that serum opsonin plays a dominant role in determining the extent of liposome clearance in vivo. Negative effect of serum on the level of liposome uptake by the liver was observed with liposomes containing CL or PA. For example, about 20% of total perfused liposomes containing PA was taken up by the liver in the absence of serum while the total liposome uptake by the liver dropped to about 10% when serum was present. A similar level of reduction (50%) in liver uptake by serum treatment was also seen in liposomes containing CL (from about 60% to 30%). The last pattern observed was that in which serum did not affect the level of liposome uptake by the perfused liver. Liposomes, belonging to this category, include those containing PI (2%), GT_{1b} (6%) and DPGS (17%).

Compared to about 10% of liver uptake for PC/Chol liposomes under the same experimental conditions [17] and using the level of liver uptake in the presence of serum for comparison, inclusion into the lipid bilayer of PI, GD, GT_{1b} and PG decreased liposome uptake by the liver. Conversely, aGM_1, PA, CL, DPGS and PE increased the liposome uptake by the perfused liver.

3.2. Liver uptake of liposomes in the presence of excess amount of liposomes with various composition

Increase in the total liposome uptake by the liver in the absence of serum for CL, PA and DPGS in Fig. 1 and by PS and DCP described in our previous report [17] would suggest that these lipids provide the liposome with higher affinity to liver cells that are involved in taking up liposomes. It would also suggest that there are specific membrane molecules (receptor) for these lipids on the cell surface. With respect to these receptors, it is possible that the binding of liposomes with different compositions involves only one type of membrane receptor. The affinity of such receptor varies with the structure of the functional lipid in the liposome bilayer. Liposomes containing the lipids with higher affinity will be taken up by liver cells more efficiently than those containing the lipids that exhibit lower affinity. Alternatively, it is also possible that there are multiple molecules involved. The level of liposome uptake by the liver cells depends on the number of binding sites available for liposome binding and the affinity between the receptor and the lipid component. To test these possibilities, a competition experiment was performed using liposomes containing PS, DCP, CL, PA and DPGS. In these experiments, ^{111}In-labeled liposomes (0.1 μmol total lipids) were mixed with 2 μmol non-labeled liposomes with the same or different lipid composition. The resulting liposomes after mixing the labeled (test liposomes) with the unlabeled (competing liposomes) were then perfused through the mouse liver and the total liver

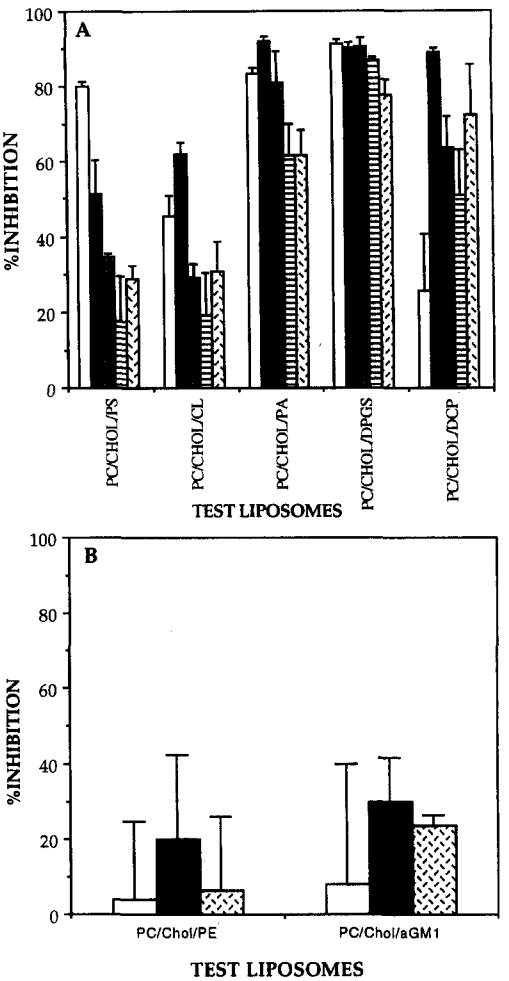

Fig. 2. Inhibition of liver uptake of liposomes by different liposome composition. ^{111}In-labeled test liposomes (0.1 μmol total lipid with composition listed on *X*-axis) were mixed with unlabeled liposomes of different compositions (2 μmol total lipids) and liver perfusion was then performed. The percent inhibition was calculated by dividing the difference between the total liver uptake in the absence and presence of competing liposomes by the liver uptake in the absence of competing liposomes. Data represent the mean ± S.D., $n = 3$–5. (A) Inhibition of serum-independent liver uptake of liposomes. Competing liposomes containing PS (1st bar, □), CL (2nd bar, ■), PA (3rd bar), DPGS (4th bar), or DCP (5th bar). (B) Inhibition of serum-dependent liposome uptake. Same experimental conditions were used as in A except that the test liposomes were preincubated with mouse serum before being mixed with the competing liposomes. Competing liposomes containing PS (1st bar, □), CL (2nd bar, ■), or DCP (3rd bar).

uptake for the test liposomes was analyzed. As seen in Fig. 2A, the effect of the excess amount of competing liposomes on liver uptake varies among the liposomes tested. Taking PS-containing liposomes as an example, the total level of liposome uptake by the liver was decreased in the presence of the excess amount of competing liposomes. As expected, about 80% (from 33% uptake to 6%) of the liver uptake for ^{111}In-labeled PS-containing liposomes was blocked when 2 μmol of same liposomes were included. Liposomes containing CL exhibited lower but significant activity in inhibiting the liver uptake of PS-containing

liposomes. Liposomes containing PA, DCP and DPGS showed similar inhibition activity with the inhibition level of about 25%. Similar inhibition pattern was also seen when CL-containing liposomes were used as the test liposomes. 60% of liver uptake was inhibited when 2 μmol of same liposomes was used. Presence of PS-containing liposomes inhibited about 50% liver uptake for CL-containing liposomes. Again, liposomes containing either DCP, PA or DPGS showed much lower inhibition activity (25% inhibition).

Different from the inhibition pattern observed in PS- and CL-containing liposomes, the liver uptake of liposomes containing PA and DPGS was more easily inhibited by the presence of competing liposomes. All liposomes tested for competition (PS-, CL- and DCP-containing liposomes) were effective in blocking the liver uptake of liposomes containing either PA or DPGS. When DCP-containing liposomes were used as the test liposomes, PS-containing liposomes showed much lower inhibition activity compared to those containing CL, PA or DPGS.

Theoretically, if the liver uptake for negatively charged liposomes as shown in Fig. 2A involves the specific receptors for negatively charged phospholipids such as PS, CL, DCP, PA and DPGS, serum-dependent liposome uptake by the liver should not be sensitive to the presence of these negatively charged liposomes because the receptors

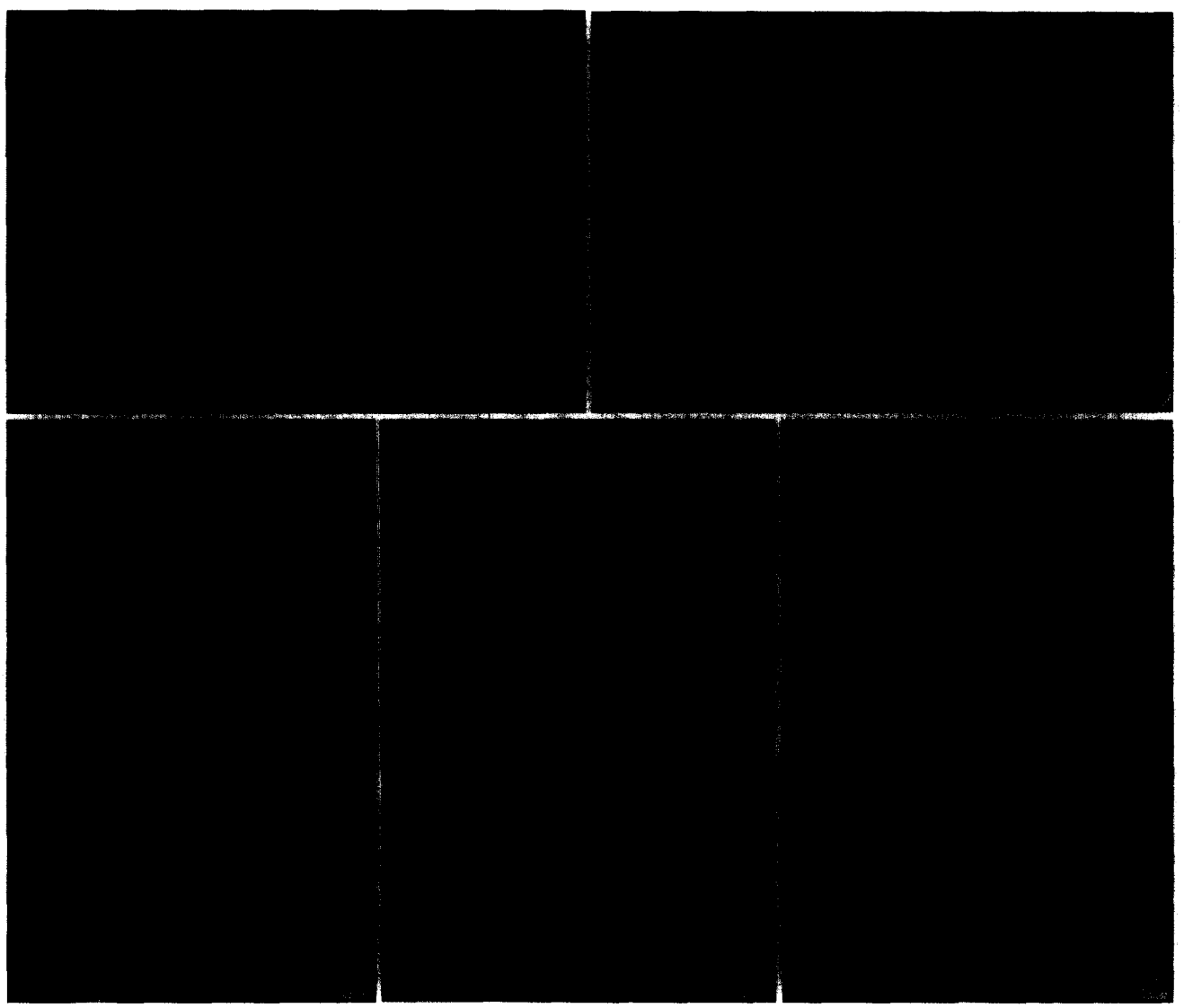

Fig. 3. Fluorescence microscopic studies of liposome localization in the liver. Fluorescence labeled polystyrene beads with a diameter of 1 μm ($1.2 \cdot 10^9$ beads/mouse) were injected to mouse via tail vein to label the Kupffer cells. A standard liver perfusion in these animals was performed with DiI-labeled liposomes (2 μmol total lipid) of various lipid compositions. Cryosections of the liver were made using Cryostat (Jung Frigocut 2800N) and examined under a fluorescence microscope. Pictures were taken using double exposure technique. Yellow color is from polystyrene beads and red color indicates the location of liposomes. Large arrow in A points the co-localization of polystyrene beads with liposomes and small arrows in D and E show different localization of polystyrene beads and liposomes. Liver was perfused with liposomes containing (A) PS, (B) CL, (C) aGM_1, (D) PE or (E) DCP. Liposomes containing aGM_1 and PE were preincubated with mouse serum for 10 min at 37°C before being perfused through the liver. The bar in C represents 20 μm.

involved in taking up these types of liposomes should be specific for opsonins bound to liposome surface. Indeed, as shown in Fig. 2B, presence of the excess amount of liposomes containing either CL, PS and DCP did not significantly affect the level of liposome uptake by the perfused liver for liposomes of PE and aGM_1. Less than 20% of liposome uptake by the liver was inhibited when 10 folds in excess of negatively charged liposomes were present.

3.3. Liver uptake of liposomes involves different cell types

To test whether liposome uptake by the liver involves different cell types, two independent approaches were taken. The first approach was based on the known information that the uptake of polystyrene beads by the liver is exclusively mediated by the Kupffer cells [22]. In these experiments, fluorescence labeled polystyrene beads were injected into animals. One hour after the injection, liver perfusion was performed using different types of liposomes labeled with DiI, a commonly used fluorescence marker for membrane studies [23], and the co-localization of both fluorescence markers was then examined using a standard fluorescence microscopic technique. It is evident in Fig. 3 that two distinct distribution patterns of the fluorescence probes were obtained. For liposomes containing PS, PE and aGM_1, DiI (red color) was well co-localized with that of Fluoresbrite YG Microspheres (Fig. 3A, C and D), suggesting that the Kupffer cells are responsible for the uptake of these liposomes. For CL- and DCP-containing liposomes, however, only partial co-localization of DiI and the fluorescence-labeled beads was observed (Fig. 3B and E). Such partial co-localization would suggest the involvement of both Kupffer and non-Kupffer cells in taking up CL- and DCP-containing liposomes.

If both Kupffer and non-Kupffer cells are involved in the uptake of CL- and DCP-containing liposomes, one would predict that elimination of Kupffer cells from the liver should only partially affect the total liposome uptake by the liver. In the same notion, for liposomes that are taken up solely by the Kupffer cells, liposome uptake by the liver should be inhibited if these cells are eliminated from liver. To test this hypothesis and confirm the conclusion drawn from data shown in Fig. 3, we have used a macrophage suicide reagent, Cl_2MBP. In these experiments, animals were pre-injected with 90 μg of Cl_2MBP encapsulated into liposomes composed of PC/Chol/PS. 24 h later, standard perfusion experiments were performed to check the total liposome uptake by the liver. As shown in Fig. 4, the total liver uptake for CL-containing liposomes decreased only about 50% in comparison to the control. The total liver uptake for DCP-containing liposomes was also partially inhibited, being about 20% in treated animals compared to about 28% in the control group. However, the liver uptake for other types of liposomes including those containing PS, PE and aGM_1 was

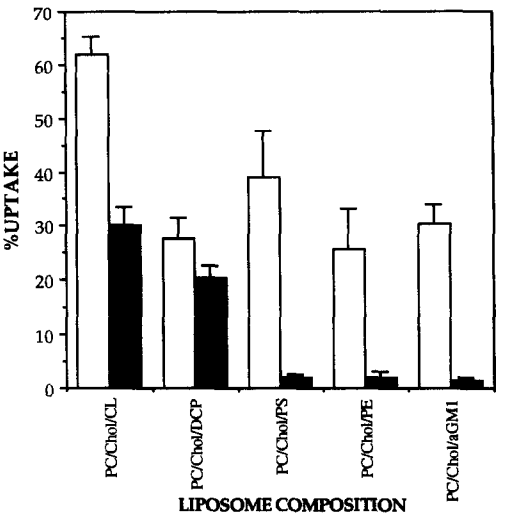

Fig. 4. Effect of Kupffer cell elimination using liposomal Cl_2MBP on the liver uptake of liposomes. Mouse was injected with 3 μmol of plain liposomes (PC/Chol/PS) or liposomes containing about 90 μg of Cl_2MBP. 24 h post injection, single-pass liver perfusion of ^{111}In-labeled liposomes was then performed on these animals according to the standard procedure. (□) treated with empty liposomes and (hatched bar) treated with Cl_2MBP-containing liposomes. Data present mean ± S.D. (n = 3–5).

completely abolished in animals pretreated with liposomal Cl_2MBP. These results strongly suggest the involvement of non-Kupffer cells for CL- and DCP-containing liposomes. The Kupffer cells, however, are solely responsible for liver uptake of PS-, PE-, and aGM_1-containing liposomes. These results support the conclusion obtained from our microscopic studies (Fig. 3).

3.4. Serum enhanced liver uptake of liposomes involves the complement system

Recent results from this laboratory using freshly collected human serum showed that the serum components that are responsible for the enhanced liposome uptake are those involved in complement activation [16]. To test whether this is also true for mouse system, freshly collected mouse serum was treated under the conditions that are known to block the complement activation pathway [24]. As shown in Fig. 5, over 90% of serum-mediated liposome uptake by the perfused liver were blocked by pretreatment of serum with either EDTA, $EGTA/Mg^{2+}$ or high temperature (56°C for 30 min). Identical results were obtained for liposomes either containing PE or aGM_1, suggesting that complement components are responsible for serum-mediated liposome uptake by the liver.

4. Discussion

The mechanisms involved in liposome clearance by the liver are fairly complicated. However, data presented in this paper and those of our previous publications [16,17]

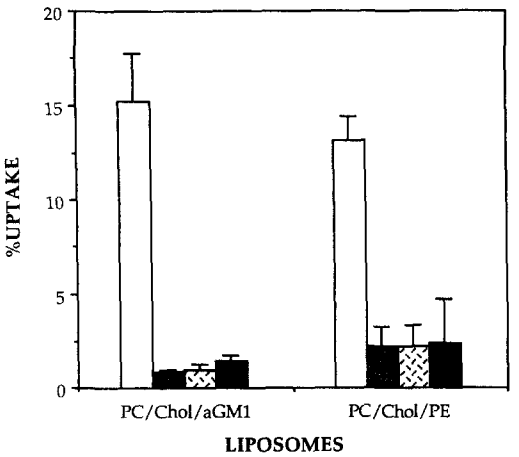

Fig. 5. Effect of blocking the complement system on the serum-mediated liposome uptake by the perfused liver. (1st bar, □) serum without treatment, (2nd bar) serum treated with EDTA, (3rd bar) serum treated with EGTA/Mg^{2+} and (4th bar) serum treated at 56°C. Data represent mean ± S.D. ($n = 3$).

show that two independent mechanisms are involved, one is mediated by serum opsonins and the other is not. For liposomes containing negatively charged phospholipids such as PS, CL, DCP, PA and DPGS, it is clear that their uptake by the liver is through a mechanism of direct recognition, without involving the serum components. In contrast, the liver uptake of liposomes containing aGM_1, PE and PG is mediated by complement components. Furthermore, while it is true that liver Kupffer cells are mainly responsible for liposome clearance, other types of liver cells may also participate in liposome uptake.

The level of liposome uptake by the liver is controlled by the structure of the hydrophilic head groups covering the liposome surface. Except for PE- and aGM_1-containing liposomes, all liposomes tested in this study are negatively charged. The fact that not all liposomes showed high liver uptake would suggest that the negatively charged surface is not the determining factor for liposome uptake by the liver. For example, liposomes containing PI, although negatively charged, exhibited a very low liver uptake regardless of the presence of serum. Furthermore, the mechanism involved in the liver uptake of negatively charged liposomes could be different. PG-containing liposomes are likely cleared from blood circulation by the liver through serum-mediated process while those containing PS, DCP, CL, PA, or DPGS are cleared through serum-independent pathway. Thus, it may not be possible to predict the rate of blood clearance of a given type of liposome solely based on its surface charge.

The high level of liver uptake in the absence of serum for liposomes containing CL, PA, DPGS (Fig. 1) and PS, DCP in our previous publication [17] appears to support the hypothesis that liver uptake of negatively charged liposomes involves the direct recognition of the negatively charged head groups (phosphate group in DCP, PA and CL, and carboxyl group of PS and DPGS) of the phospholipids by membrane receptors of the liver cells. One common feature for these liposomes is that the negatively charged head groups of these negatively charged phospholipids are directly exposed to the liposome surface. The reason for the low level of liver uptake for PG-containing liposomes in the absence of serum, even though they contain negatively charged phosphate group, is probably because the phosphate groups of PG on liposome surface are not recognized by these membrane receptors. This may be due to the steric hindrance provided by glycerol head group on PG. Low level of liver uptake for PI-containing liposomes may be due to the same mechanism. In the same notion, 3-fold increase in liver uptake for GT_{1b}-containing liposomes, compared to the level for PI-containing liposomes, would suggest that there are certain amounts of negatively charged carboxyl groups on GT_{1b}-containing liposomes that are available for recognition. Such low level of exposure, although sufficient to result in a decreased liposome circulation time in vivo [10], is not enough to result in a high level of liver uptake in our perfusion system. Such explanation is consistent with the hypothesis that the hindrance effect of the sugar moieties on the negative charge plays a key role in the prolongation of liposome circulation time in blood [10].

It is evident from Fig. 1 that the clearance of liposomes containing PE and aGM_1 in vivo involves serum opsonins. Such serum mediated liposome uptake has been reported by many laboratories (for review, see [25]) including ours [16,17,26,27]. The loss of serum activity in increasing the liver uptake for PE- and aGM_1-containing liposomes by EDTA and EGTA/Mg^{2+} would suggest that activation of complement system by these liposomes is through the classic pathway. Activation of complement system by PE [28] and other glycolipid such as cetyl mannoside [29] in the form of liposomes has been reported previously. It has also been reported that complement system is involved in the clearance of PG-containing liposomes in vivo [30]. Such conclusion also seems to be true for other types of liposomes in different animal systems [16]. It is not clear at this moment, however, whether the complement activation by these lipids involves the antibodies specific to these lipids. Limited evidence suggests that C3b of the complement system is responsible for the serum mediated liposome uptake by the liver [25,27,31].

In addition to the fact that serum enhances the liver uptake of PE- and aGM_1-containing liposomes, data in Fig. 1 show that serum decreases the level of liver uptake for CL- and PA-containing liposomes. A similar phenomenon has also been seen for PS-containing liposomes [17]. Since such negative effect by the serum was only observed in liposomes that exhibit high liver binding in the absence of serum and it is not sensitive to the treatment designed to inactivate the complement system [16], it is highly possible that such effect is resulted from the nonspecific binding of serum proteins to the surface of these liposomes. Under such a condition, upon the exposure of

liposomes to the serum, some serum proteins will non specifically adsorb to the liposome surface and provide some level of steric hindrance that can interfere with the binding of liposomes to the cells through the specific membrane receptor. The types and amount of serum proteins that can bind to the liposome surface will depend on the surface properties of the liposomes, thus some negatively charged liposomes (CL, PA liposomes, Fig. 1) show significant reduction in liposome uptake by the liver and some liposomes such as those containing DPGS do not when serum components are present. Different levels of protein binding to liposomes of various lipid compositions have been well documented in the literature [32,33].

Data in Figs. 3 and 4 suggest that, while it is true that liver Kupffer cells are the major cell type in the liver that are responsible for liposome uptake, non-Kupffer cells may also participate in the uptake of liposomes containing CL or DCP. To our knowledge, this is the first time, using large sized liposomes, the involvement of non-Kupffer cells in the liposome uptake is demonstrated. Taking the fact that the diameter of the liver fenestration is about 100 nm [34] and the average diameter of our liposomes is above 300 nm, it is unlikely that the hepatocytes, which are located outside the blood vessels, are involved. While it is possible that these non-Kupffer cells are some subclasses of macrophages that do not normally take up liposomes, it is also possible that other liver cells such as the endothelia cells forming the capillary wall of the blood vessel are involved.

While it is evident that different types of liver cells are involved in liposome uptake, the molecules on the cell surface responsible for liposome uptake are still not clear. The fact that a large proportion of liver uptake for liposomes containing PA or DPGS (Fig. 2) can be inhibited by excess amount of liposomes containing either CL, PS or DCP would suggest that these molecules involved in binding of PA and DPGS are shared by CL, PS and DCP. The reduced effectiveness of these two types of liposomes in blocking the uptake of liposomes containing CL, PS and DCP indicates that the affinity of the receptors involved in the binding of PA and DPGS liposomes is lower. An alternative explanation for this observation, even though less likely, is that the number of binding sites for these two types of liposomes is less than those for other liposomes. In this case, different types of liposomes are taken up through different molecules. The reason that one type of liposomes can block the binding of the other is because the binding of competitive liposomes when they are in excess will sterically hinder the binding of the other. Under such circumstances, the cell surface is basically covered by the competing liposomes and there may not be enough room left on the cell surface for the other type of liposome to bind, thus they are "competed out" from binding to the liver, resulting in a low liver uptake. The higher efficiency of CL-containing liposomes as shown in Fig. 2 in inhibiting the liver uptake of other types of liposomes is presumably related to the double negatively charged phosphate group in each molecule of CL. Theoretically, among the negatively charged lipids tested, CL may be the only one that contains two directly exposed negatively charged phosphate groups. Nevertheless, our data suggest the existence of multiple receptors that are likely involved in liposome clearance.

While it is evident from our results summarized in this and previous report [17] that different receptors are most likely involved in the uptake of liposomes by the liver, the identity and physiological functions of these receptors are still not known. Concerning the fact that liver cells, especially Kupffer cells, are part of immune defense system that keep abnormal substances and cells out of circulation, it is, therefore, possible that these receptors are involved in the binding of these abnormal substances. For example, it has been reported that PS is exclusively located in the inner leaflet of the cell membrane. Appearance of PS on the outer leaflet of the cell membrane will result in a quick clearance of the cells from the blood circulation by the RES. The mechanisms of such clearance may be similar, if not identical, to that of the clearance for PS-containing liposomes as discussed in this report. Studies to explore the physiological functions of the receptors involved in the uptake of other types of liposomes such as those containing CL, PA, DCP and possibly others are yet to be performed.

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References

[1] Lasic, D.D. (1993) in Liposomes: From Physics to Applications, Elsevier, New York.
[2] Gregoriadis, G. (1988) in Liposomes as Drug Carriers: Recent Trends and Progress, John Wiley, New York.
[3] Juliano, R.L. (1988) Adv. Drug Deliv. Rev. 2, 31–54.
[4] Lasic, D.D. and Papahadjopoulos, D. (1995) Science 267, 1275–1276.
[5] Huang, S.K., Stauffer, P.R., Hong, K., Guo, J.W., Phillips, T.L., Huang, A. and Papahadjopoulos, D. (1994) Cancer Res. 54, 2186–2191.
[6] Gabizon, A., Catane, R., Uziely, B., Kaufman, B., Safra, T., Cohen, R., Martin, F., Huang, A. and Barenholz, Y. (1994) Cancer Res. 54, 987–992.
[7] Wu, N.Z., Da, D., Rudoll, T.L., Needlham, D., Whorton, A.R. and Dwhirst, M.W. (1993) Cancer Res. 53, 3765–3770.

[8] Huang, S.K., Mayhew, E., Gilani., S., Lasic, D.D., Martin, F.J. and Papahadjopoulos, D. (1992) Cancer Res. 52, 6774–6781.
[9] Senior, J.H. (1998) Crit. Rev. Ther. Drug Carr. Syst. 3, 123–193.
[10] Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 85, 6949–6953.
[11] Juliano, R.L. and Stamp, D. (1975) Biochem. Biophys. Res. Commun. 63, 651–658.
[12] Liu, D., Mori, A., and Huang, L. (1992) Biochim. Biophys. Acta 1104, 95–101.
[13] Klibanov, A.L., Maruyama, K., Torchilin, V.P. and Huang, L. (1990) FEBS Lett. 268, 235–237.
[14] Allen, T.M., Hausen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Biochim. Biophys. Acta 1066, 29–36.
[15] Senior, J., Delgardo, C., Fisher, D., Tilcock, C. and Gregoriadis, G. (1991) Biochim. Biophys. Acta 1062, 77–82.
[16] Liu, D., Hu, Q. and Song, Y.K. (1995) Biochim. Biophys. Acta 1240, 277–284.
[17] Liu, F. and Liu, D. (1996) Biochim. Biophys. Acta 1278, 5–11.
[18] Kabalka, G., Buonocore, E., Hubner, K., Moss, T., Norley, N. and Huang, L. (1987) Radiology 163, 255–258.
[19] Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim Biophys Acta 812, 55–65.
[20] Van Rooijen, N. (1989) J. Immunol. Methods 124, 1–6.
[21] Wake, K., Decker, K., Kirn, A., Knock, D.L., McCuskey, R.S., Bouwens, L. and Wisse, E. (1989) Int. Rev. Cytol. 118, 173–229.
[22] McCuskey, R.S. (1993) in Molecular and Cell Biology of the Liver (LeBouton, A.V., ed.), pp. 407–427, CRC Press, Boca Raton, FL.
[23] Classen, E. (1992) J. Immunol. 147, 231–240.
[24] Mayer, M.M. (1961) in Experimental Immunology (Kabat, E.A. and Mayer, M.M., eds.), pp. 133–240, Charles C. Thomas, Springfield.
[25] Patel, H.M. (1992) Crit. Rev. Ther. Drug Carr. Syst. 9, 39–90.
[26] Liu, D., Liu, F. and Song, Y.K. (1995) Biochim. Biophys. Acta 1235, 140–146.
[27] Liu, D., Liu, F. and Song, Y.K. (1995) Pharm. Res. 12, 508–512.
[28] Mold, C. (1989) J. Immunol. 143, 1663–1668.
[29] Funato, K., Yamashita, C., Kamada, J., Tominaga, S. and Kiwada, H. (1994) Pharm. Res. 11, 372–376.
[30] Loughrey, H.C., Bally, M.B., Renish, L.W. and Cullis, P.R. (1990) Thromb. Haemost. 64, 172–176.
[31] Harashima, H., Sakata, K., Funato, K. and Kiwada, H. (1994) Pharm. Res. 11, 402–406.
[32] Juliano, R.L. and Lin, G. (1980) in Liposomes and Immunology (Six, H. and Tom, B., eds.), pp. 49–66, Elsevier, Amsterdam.
[33] Chonn, A., Semple, S.C. and Cullis, P.R. (1992) J. Biol. Chem. 267, 18759–18765.
[34] Wisse, E. (1970) J. Ultrastruct. Res. 31, 125–150.