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Lipid transfer protein transports compounds from lipid nanoparticles to plasma lipoproteins

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Abstract

Nanometer-sized lipid emulsion particles with a diameter of 25–50 nm, called Lipid Nano-Sphere (LNS®), are expected as a promising drug carrier to show prolonged plasma half-life of an incorporating drug. In terms of successful drug delivery using LNS, a drug should be incorporated into the lipid particles and remain within the particle, not only in the formulation in vitro but also after administration into the systemic blood circulation. In this study, we showed that phospholipids and some water-insoluble molecules also moved from lipid particles to plasma lipoproteins or albumin in serum and plasma half-lives of these compounds did not reflect that of the drug carriers. It was suggested that phospholipid or its derivative were transferred from LNS particles to plasma lipoproteins by lipid transfer proteins (LTP) in the circulation. These phenomena leaded to unsuccessful delivery of the drug with lipid–particulate drug carriers. On the other hand, lipophilic derivatives with cholesterol pro-moiety tested in this study were not released from LNS particles and showed prolonged plasma half-lives. Lipophilicity is known to be an important parameter for incorporating drugs into lipid particles but substrate specificity for LTP seems to be another key to success promising drug design using lipid emulsion particulate delivery system.
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Keywords: Lipid emulsion; Nanoparticle; Lipid transfer protein; LNS; Drug delivery

1. Introduction

Numerous drug carriers for improved drug delivery, such as liposomes and lipid emulsions, have been proposed to modify the disposition of pharmacologically active agents. Most exogenous colloidal drug carriers have difficulty achieving successful in vivo drug targeting. The critical problem seems to be the apparent non-specific uptake of the carrier particles as a foreign substance by tissues that have developed a reticuloendothelial system, such as the liver and spleen.

We developed a lipid emulsion particle, called Lipid Nano-Sphere (LNS®), which is composed of phospholipids and simple lipids and has a similar diameter (25–50 nm) to endogenous plasma lipoproteins (Seki et al., 1988, 1994; Sugiyama and Seki, 1991). A conventional fat emulsion for intravenous nutrition, called Lipid Microsphere (LM, $d = 200$–300 nm) is already used clinically as a drug carrier (Mizushima, 1996; Yokoyama and Watanabe, 1996). LNS and LM can be manufactured with the same lipids such as soybean oil and egg lecithin. The major physical difference be-

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tween LNS and LM is their particle diameter. LNS showed a prolonged plasma half-life of an incorporating drug, such as dexamethasone palmitate and amphotericin B, with superior efficacy of delivery of the pharmacological active compounds to inflamed sites (Fukui et al., 2003a; Seki et al., 2004a).

In terms of successful drug delivery using these lipid particles as a carrier, a drug should be incorporated into lipid emulsion particles and remain in the particle not only in the formulation (in vitro) but also after introduction into the circulation (in vivo). There are many approaches for the successful incorporation of lipophilic drug derivatives into liposomes and emulsions. For example, numerical lipophilic mitomycin C derivatives were synthesized and their characteristics examined (Sasaki et al., 1983). Most of those studies showed that lipophilicity or the lipophilic index is the key, and Takino et al. (1994) reported that highly lipophilic compounds with a $\log PC_{oct}$ of more than 9 were suitable for successful incorporation into lipid emulsion particles. However, the key factors for suitable drug design are not fully understood.

In this study we have focused on drug dissociation from LNS particles in rat serum because it is considered that lipid emulsion particles have dynamic physiological lipid/lipoprotein metabolism in the blood circulation after intravenous injection. In the blood stream, exogenous triglyceride-rich lipid particles undergo lipolysis by lipoprotein lipase (LPL) in a particle size-dependent manner and the resulting particles are taken up by the liver in a similar way with chylomicron/VLDL (Iriyama et al., 1987; Mahley, 1988; Morita et al., 2003; Rensen et al., 1997; Wasan and Cassidy, 1998; Yamamoto et al., 2003). Namely, intravenously administered lipid particles can be assumed to have an opportunity to interact with plasma lipoproteins, proteins, peripheral cells, etc.

Lipid transfer proteins (LTP), such as phospholipid transfer protein (PLTP, LTP-I) and cholesterol ester transfer protein (CETP, LTP-II) were in plasma and tissues (Guyard-Dangremont et al., 1998). They are involved in lipid and lipoprotein metabolism and are responsible for the facilitated transfer of phospholipids, cholesterol esters, and triglycerides among lipoprotein particles. For example, some directional transportations of lipids among lipoproteins are known, i.e. CETP facilitates transfer of cholesterol esters from HDL to VLDL/LDL. If a lipophilic drug derivative in an exogenous lipid particle is recognized by LTP, the drug may be removed from the lipid particle and moved to plasma lipoprotein. This may give serious influence on the efficacy of drug delivery or targeting with an exogenous lipid–particulate system.

2. Materials and methods

2.1. Materials

Purified egg lecithin and soybean oil for parenteral use were purchased from Q.P. Corporation, Tokyo, Japan and Ajinomoto Co., Inc., Tokyo, Japan, respectively.

L-α-Dipalmitoyl [2-palmitoyl-9,10-^{3}H(N)] phosphatidylcholine (^{3}H-DPPC), cholesteryl [cholesteryl-4-^{14}C] oleate (^{14}C-CO), and cholesteryl [cholesteryl-1,2-^{3}H(N)] hexadecyl ether (^{3}H-CHE) were purchased from NEN, Wilmington, DE, USA. Cholesteryl anthracene-9-carboxylic acid (CA) was of Wako Pure Chemical Industries, Ltd., Osaka Japan. *N*-(1-Pyrenesulfonyl)-1, 2-hexadecanoyl-sn-glycero-3-phosphoethanolamine (PP) and 12-(9-anthroyloxy)stearic acid (SA) were purchased from Molecular Probe, Inc., Eugene, OR, USA and the fluorescent markers were used without purification. Three lipophilic mitomycin C derivatives, cholesteryloxycarbonyl mitomycin C (Chol-MMC), cetyloxycarbonyl mitomycin C (C16-MMC), and nonyloxycarbonyl mitomycin C (C9-MMC), were synthesized in our laboratory according to the literature (Sasaki et al., 1983). Their structure and purity were confirmed by elemental and spectral (NMR) analyses. Their molecular weights and partition coefficients ($\log PC_{oct}$) calculated by Hansch and Leo's fragment methods using a computer program are: CA (MW, 590.88; log PC, 14.74), PP (MW, 956.26; log PC, 18.65), SA (MW, 488.70; log PC, 10.98), Chol-MMC (MW, 746.97; log PC, 9.19), C16-MMC (MW, 602.76; log PC, 7.20), C9-MMC (MW, 504.58; log PC, 3.50). All other chemicals used in this study were of reagent or analytical grade.

2.2. Preparation of lipid emulsions

The compositions and physicochemical characteristics of LNS and LM incorporating various compounds were described previously (Fukui et al., 2003a; Seki

et al., 2004b). LNS was composed of equal amounts of purified egg lecithin and soybean oil. LM was also composed of the same ingredients and the ratio of egg lecithin to soybean oil was 0.12 to 1 by weight. The total lipid concentration in both lipid emulsions was 10% (w/v) and the aqueous phase of the lipid emulsions was 0.24 M glycerin. Briefly, the mixtures were homogenized and emulsified sufficiently in 0.24 M glycerin with a Microfluidizer® 110-Y or 110-E/H (Microfluidics International Corporation, Boston, MA, USA) at 100 MPa for LNS and at 50 MPa for LM with a Y-type Interaction Chamber by applying 0.2–5% of backpressure (Saheki and Seki, 1998). Handa and his coworkers reported that they needed to remove contaminating vesicles from their lipid emulsion preparations by ultracentrifugation to make homogenous emulsion particles (Morita et al., 2003). On the other hand, we could make highly homogenous preparations without any purification. Our preparations included not phospholipid bilayer vesicles but a small amount of discoidal small particles in some cases as shown in our previous reports (Fukui et al., 2003a; Seki et al., 2004a). Therefore, LNS and LM in this study were used without further purification. A fluorescent lipid or mitomycin derivative listed above was incorporated into the emulsions at a concentration of 0.25 mg/ml.

The particle size of the lipid emulsions was determined by a laser dynamic light scattering particle sizer, DLS-700, Otsuka Electronics, Inc., Osaka, Japan, after diluting the emulsion with distilled water (Zhang and Kirsch, 2003). LNS and LM were in the range of 25–50 and 200–300 nm in diameter, respectively. All lipid emulsions were stable throughout this study even at room temperature when they were sealed in ampoules with nitrogen gas. There were no free marker compounds found in the all preparations determined by ultrafiltration. However, slight chemical degradation of C9-MMC and C16-MMC, hydrolysis to free mitomycin C, was found in the preparations 1 week after manufacture, so they were prepared weekly.

2.3. Animal experiments

Male Sprague–Dawley rats were purchased from Japan S.L.C. (Hamamatsu, Japan) and used for all experiments at 6–8 weeks of age without fasting. LNS or LM incorporating a radio-, fluorescent-labeled lipid, or mitomycin C derivative was administered intravenously to rats at a dose of 200 mg of lipids/kg. Venous blood (0.2 ml) was corrected and the plasma concentration of radioactivity, of the fluorescent lipid or of the mitomycin derivative after intravenous administration was determined with a liquid scintillation counter, a fluorescent photometer (Ex 380 nm, Em 464 nm), or a spectrophotometer (at 380 and 560 nm), respectively. After chloroform:methanol (1:1, v/v) co-solvent extraction from the plasma, the concentration of the fluorescent lipid, or of the mitomycin C derivative was determined using a calibration curve to correct the extraction efficiency.

Serum (80 μl) was also obtained from the venous blood and applied on a Sepharose CL-6B column for size-exclusion chromatography as described below.

2.4. Size-exclusion chromatography

Fifty microliters of LNS incorporating a radiolabeled lipid, a fluorescent lipid, or a mitomycin C derivative was mixed and incubated with 1 ml of fresh serum obtained from normal rats at 37 °C for 10 min. To evaluate the transfer of these marker compounds from LNS to serum proteins and lipoproteins, the mixture (80 μl) was applied onto a Sepharose CL-6B column (1 cm × 45 cm; Amersham Biosciences, Piscataway, NJ, USA) equilibrated with phosphate-buffered saline. The sample was eluted with the same buffer at a flow rate of 0.22 ml/min, and 8-ml fractions were collected. The entire chromatographic separation was completed in 4 h. LNS eluted in the same fraction as very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL). The determination of radioactivity, fluorescent intensity, or mitomycin C derivatives in the fractions was described above. Lipoprotein-free serum was obtained by ultracentrifugal floatation (100,000 × g at 4 °C for 48 h) at a density of 1.21 with sodium bromide followed by dialysis against phosphate-buffered saline. Serum LPL activity was suppressed by the addition of NaCl to the serum at a final concentration of 1 M (Korn, 1955). PLTP in the serum was suppressed by heating at 54 °C for 30 min (Tollefson et al., 1988). These in vitro experiments were done using three separate sera.

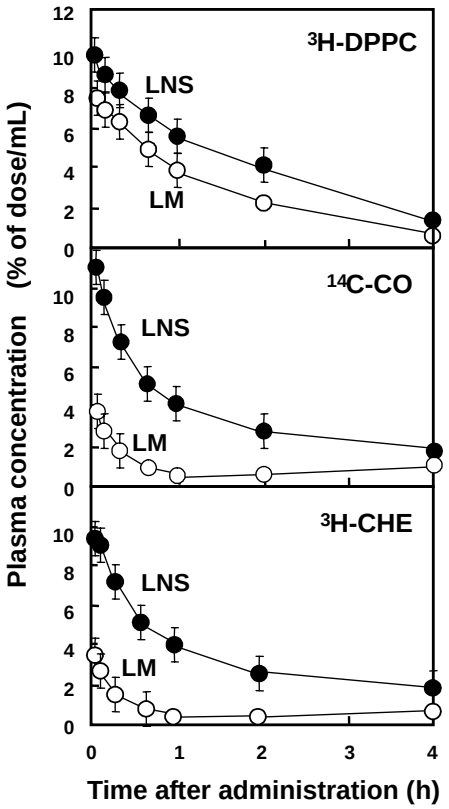

Fig. 1. Plasma concentration profiles of radioactivity after the intravenous administration of LNS and LM incorporating radiolabeled lipids to rats. Each point represents the mean ± S.D. of three rats. ^{3}H-DPPC. L-α-Dipalmitoyl [2-palmitoyl-9,10-^{3}H(N)] phosphatidylcholine; ^{14}C-CO, cholesteryl [cholesteryl-4-^{14}C] oleate; ^{3}H-CHE, cholesteryl [cholesteryl-1,2-^{3}H(N)] hexadecyl ether.

3. Results and discussion

3.1. Plasma levels of radiolabeled lipids incorporating into LNS and LM

We showed prolonged plasma half-lives of LNS with several compounds such as dexamethasone palmitate (Seki et al., 2004a), amphotericin B (Fukui et al., 2003a) and the differences were explained with hepatic clearance of the lipid particles.

Fig. 1 shows the plasma concentration profiles of radioactivity after the intravenous administration of LNS or LM incorporating radiolabeled lipids to rats. The plasma profiles of ^{14}C-CO and ^{3}H-CHE administered with LM were different with LNS. On the other hand, only slight difference in the plasma profiles of LNS and LM was found in ^{3}H-DPPC. Additionally, the plasma level of ^{3}H-DPPC administered with LM was over to those of ^{14}C-CO-LNS and ^{3}H-CHE-LNS. In our previous report (Seki et al., 2004c), the plasma clearance of triglycerides was much faster after administration of LM than of LNS. However, the slope for decreasing the plasma levels of phospholipids after administration of LM was almost the same with that of LNS. These results and Fig. 1 suggest that phospholipid molecule composing lipid particles behaved independently with lipid emulsion particles in the circulation. It is also suggested that plasma profiles for all components should be evaluated to have better information on the behavior of lipid–particulate delivery system. If a drug was incorporated into LNS or LM and behaved in the same way with ^{3}H-DPPC, there would be no advantage of a drug delivery system using lipid particles with different characteristics.

3.2. Plasma levels of fluorescent lipids incorporated into LNS and LM

Fig. 2 shows the plasma concentration profiles of fluorescent lipids after the intravenous administration of LNS and LM incorporating fluorescent lipids to rats. Among these three compounds, the plasma concentration of a cholesterol derivative (CA) administered with LM was most different with LNS in rats. The data with CA seems to be reflected the characteristics of the lipid particles. On the other hand, small differences in the plasma profiles of LNS and LM were found in the phospholipid derivative (PP) and fatty acid derivative (SA), as in a similar to that of ^{3}H-DPPC. It was suggested that these lipid molecules, phospholipid and fatty acid derivatives, behaved independently in the circulation with exogenous lipid emulsion particles. It is well known that serum albumin binds fatty acids with high affinity. It is possible that the fatty acid derivative have much affinity to serum albumin and could move from lipid emulsion particles to albumin in the circulation.

3.3. Plasma levels of lipophilic mitomycin C derivatives incorporated into LNS

Fig. 3 shows the plasma concentration profiles of lipophilic mitomycin C derivatives after intravenous

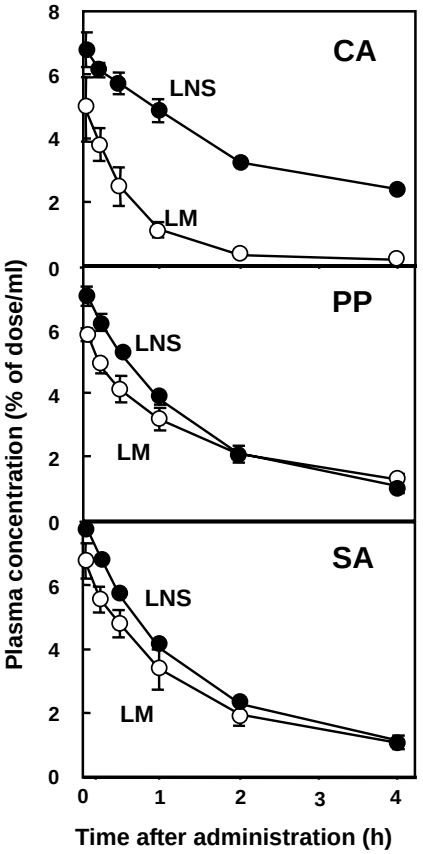

Fig. 2. Plasma concentration profiles of fluorescent intensity after the intravenous administration of LNS and LM incorporating fluorescent lipids to rats. Each point represents the mean ± S.D. of three rats. CA, cholesteryl anthracene-9-carboxylic acid; PP, *N*-(1-pyrenesulfonyl)-1,2-hexadecanoyl-sn-glycero-3-phosphoethanolamine; SA, 12-(9-anthroyloxy)stearic acid.

administration with LNS. The plasma profile of the cholesterol derivative (Chol-MMC) administered with LNS was highest among these three compounds. The other two compounds, C16-MMC and C9-MMC administered with LNS did not show a prolonged plasma profile. Takino et al. (1994) reported that highly lipophilic drugs with log PC_{oct} values larger than 9 show the same behavior as lipid emulsion particles in the circulation. They showed that log PC_{oct} is an important parameter of drugs suitable for a parenteral delivery system using lipid emulsion particles. As shown in Section 2, C9-MMC and C16-MMC have lower log PC_{oct} values than 9.

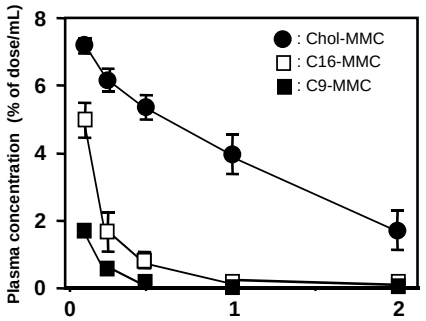

Fig. 3. Plasma concentration profiles of lipophilic mitomycin C derivatives after the intravenous administration of LNS and LM incorporating the lipophilic mitomycin C derivatives to rats. Each point represents the mean ± S.D. of three rats. Chol-MMC, cholesteryloxycarbonyl mitomycin C; C16-MMC, cetyloxycarbonyl mitomycin C; C9-MMC, nonyloxycarbonyl mitomycin C.

3.4. Size-exclusion chromatography

Figs. 4 and 5 are the representative elution patterns for the six compounds and show in vitro transfer of various compound from LNS particles to serum proteins. LNS particles incorporating the compounds were incubated with rat serum in test tubes. The serum proteins and lipid particles were separated by size-exclusion chromatography using a Sepharose CL-6B. LNS particles applied with the buffer were eluted from the column with more than 95% recovery into the fractions corresponding to VLDL and LDL.

In Fig. 4, more than 85% of applied CA was recovered in the VLDL/LDL/LNS fraction with minor tailing to the HDL fraction in three separate incubations followed by chromatographic separations. There was no elution of CA in the albumin and free fractions. On the other hand, PP was eluted in two peaks in the VLDL/LDL/LNS (43%) and HDL (55%) fractions in triplicate experiments. Most SA (81%) eluted in the albumin fraction and minor amount of SA was found in the VLDL/LDL/LNS fraction. These data indicated that PP moved from LNS to lipoproteins, especially to HDL, and SA moved to serum albumin. As discussed above, fatty acids have a high affinity to serum albumin. It is reasonable that SA is released from LNS and binds to albumin.

As shown in Fig. 5, similar to Fig. 4, only Chol-MMC was recovered completely in the VLDL/LDL/LNS fraction among these three lipophilic mitomycin

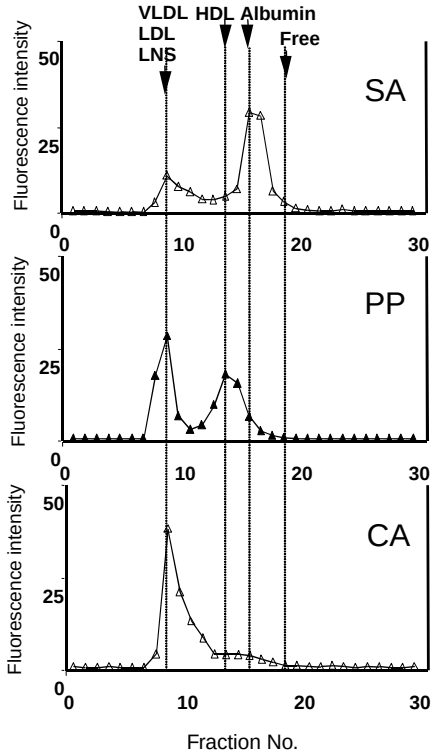

Fig. 4. Size-exclusion chromatography of LNS incorporating lipophilic compounds. The LNS preparations were incubated with rat sera followed by applying on a Sepharose CL-6B column to separate plasma proteins. Collected fractions were analyzed by fluorescent or optical density. CA, cholesteryl anthracene-9-carboxylic acid; PP, *N*-(1-pyrenesulfonyl)-1,2-hexadecanoyl-sn-glycero-3-phosphoethanolamine; SA, 12-(9-anthroyloxy)stearic acid.

C derivatives. C16-MMC was found in all three fractions and C9-MMC moved to the albumin fraction.

In vivo experiments in rats administered intravenously with LNS were also done for these compounds. Analyses for the sera obtained from the rats were done in the same way using a Sepharose CL-6B column. Our previous report using the same chromatographic technique for amphotericin B incorporated into LNS showed that no transfer to HDL or albumin was found in plasma obtained after intravenous administration to rats (Fukui et al., 2003b). As in a similar to amphotericin B, only a peak for CA and Chol-MMC in the VLDL/LDL/LNS fraction was found in the sera obtained from rats up to 2 h after administration. However, PP was found in two peaks in

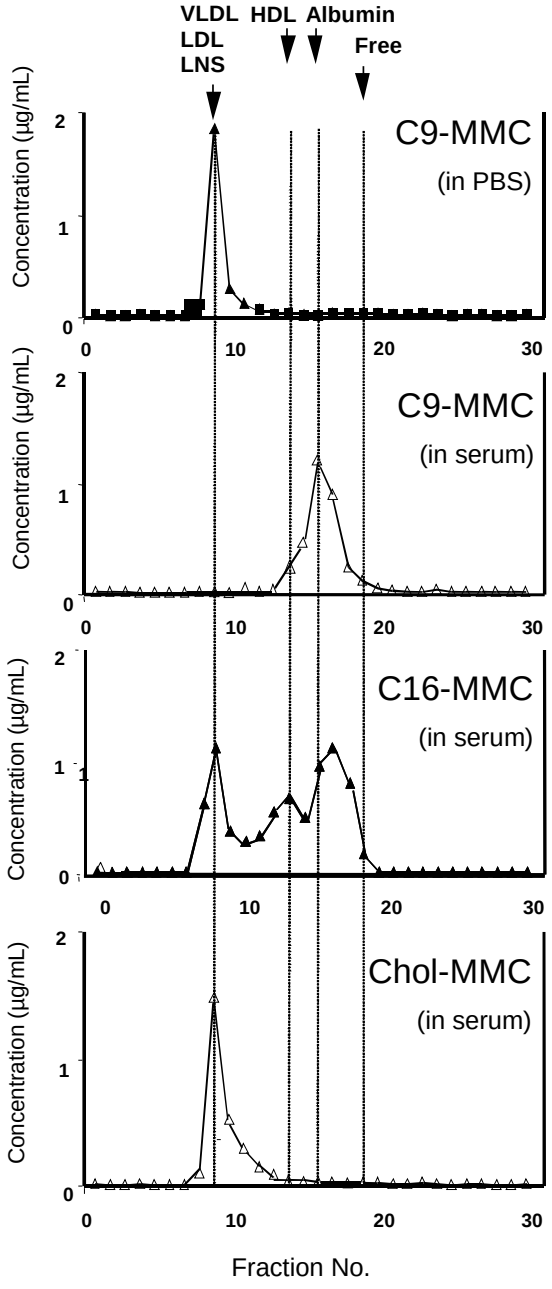

Fig. 5. Size-exclusion chromatography of LNS incorporating lipophilic mitomycin C derivatives. The LNS preparations were incubated with rat sera or PBS followed by applying on a Sepharose CL-6B column to separate plasma proteins. Collected fractions were analyzed by optical density. Chol-MMC, cholesteryloxycarbonyl mitomycin C; C16-MMC, cetyloxycarbonyl mitomycin C; C9-MMC, nonyloxycarbonyl mitomycin C.

the VLDL/LDL/LNS and HDL fractions. SA and C9-MMC were found in a peak in the albumin fraction. These in vivo results were corresponded with that of the in vitro experiments as shown in Figs. 4 and 5. But in the in vivo experiment for C16-MMC, only one peak was found in the albumin fraction for the serum obtained immediately (5 min) after intravenous injection of C16-MMC with LNS.

As discussed above, $\log PC_{oct}$ is an important parameter of drugs suitable for a parenteral delivery system using lipid emulsion particles. As shown in Section 2, C9-MMC and C16-MMC have lower $\log PC_{oct}$ values than 9. These compounds were released from the lipid particles not in a buffer but in serum. These phenomena meant that these compounds with relatively lower lipophilicity have sufficient affinity to lipid particle in a buffer but they have much affinity to albumin in biological fluid. Though SA has $\log PC_{oct} = 10.98$, it was released from LNS particles and moved to albumin in biological fluid. SA could be recognized as a fatty acid derivative with high affinity to albumin. It is possible that lower affinity to serum proteins such as albumin is an important character to prevent drug release from lipid particles. This may also be the key to success for deriving of drugs suitable for a lipid-based drug carrier system.

On the other hand, though PP has a highest $\log PC_{oct}$ value (18.65), it was released from LNS particles and moved not to albumin but to the HDL fraction (Fig. 4), and only slight difference was found in their plasma profiles administered with LNS and LM (Fig. 2). It was reported that warfarin and its lipophilic derivative, warfarin hexadecyl ether, were distributed among plasma proteins. Warfarin has an affinity to albumin but the lipophilic derivative was not distributed to albumin but to plasma lipoproteins (Tokui et al., 1995). An additional key for transfer of compounds among exogenous lipid particles and plasma lipoproteins should be considered to explain these phenomena.

3.5. Phospholipid transfer protein mediated the transport of lipophilic compounds

Nevertheless, PP was the most lipophilic compound with the highest molecular weight in this study, PP moved from LNS to HDL in serum (Fig. 4). We hypothesized two mechanisms for this transport of PP from LNS particles to the HDL fraction. One major possibility was that LTP mediated the transport of PP from LNS to HDL particles. The second possibility was that LPL mediated formation of HDL-like particles from LNS particles in the HSPG/LRP pathway (Untracht, 1982; Beisiegel et al., 1989; Iriyama, 1996). It is known that during the lipolysis of chylomicron or VLDL, phospholipid molecules form discoidal particles called nascent HDL. If LNS follow this pathway, PP may move to newly formed discoidal particles during the hydrolysis of triglycerides by LPL in the HSPG/LRP pathway. For the two possibilities, we tried to inhibit the activities of PLTP and LPL in serum by heating at 54 °C for 30 min and with 1 M NaCl, respectively.

Fig. 6 shows representative elution patterns of PP-LNS incubated with the sera as described above. As expected, PP was not found in the HDL fraction by the incubation of LNS with lipoprotein-free serum. PP moved to the HDL fraction under the inhibiting condition of LPL activity by 1 M NaCl. These data indicate that no HDL-like particles were formed newly and endogenous HDL particles were essential for the transport of PP as an acceptor. It is known that PLTP can be inactivated easily by heating. Using heated serum, no transport of PP to the HDL fraction was observed. This result suggested that the transport of PP from LNS to HDL particles was facilitated by PLTP, though several proteins were known to be inactivated by the heat-stress such as complements, lecithin–cholesterol acyltransferase, etc. (Sweeny and Jonas, 1985).

From the results in this study, all component molecules composing the lipid emulsion particles were assumed to behave dynamically and separately in their relation to endogenous lipids in the blood circulation in vivo (Tall, 1986). It is known that LTP including PLTP and CETP, has a wide substrate specificity (Kwong and Wasan, 2002; Sweeny and Jonas, 1985). An approach using lipophilic derivation with cholesterol pro-moiety seemed to be suitable for lipid–particulate delivery system, because all cholesteryl derivatives tested were released hardly from LNS particles. These compounds include CHE, CO, CA, and Chol-MMC in this study. CETP facilitates the transfer of cholesteryl esters from HDL to VLDL/LDL with a reciprocal transfer of triglycerides. This functional direction may be important to understand drug transfer, i.e. VLDL/LDL including probably LNS particles can not release but receive

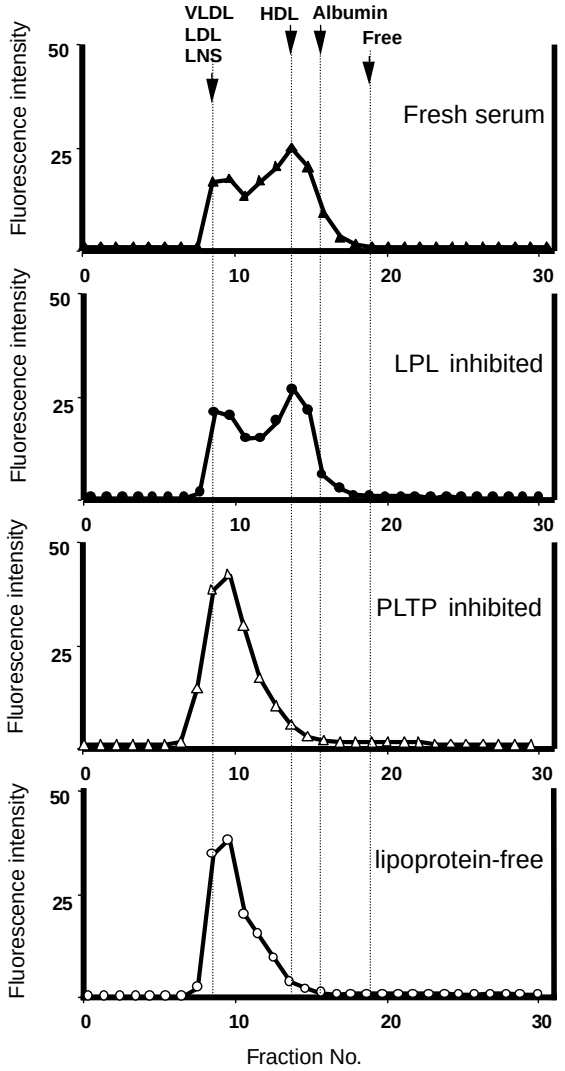

Fig. 6. Size-exclusion chromatography of LNS incorporating *N*-(1-pyrenesulfonyl)-1,2-hexadecanoyl-sn-glycero-3-phosphoethanolamine incubated with various sera. LNS-PP was incubated with various rat sera followed by applying on a Sepharose CL-6B column to separate plasma proteins. Collected fractions were analyzed by fluorescent intensity. Lipoprotein-free serum was obtained by ultracentrifugation at $d = 1.21$. Activities of PLTP and LPL in serum were suppressed by heating at 54 °C for 30 min and with 1 M NaCl, respectively.

cholesterol esters in the physiological lipoprotein metabolism. According to this, lipophilic derivation of drug for lipid based drug delivery should be targeted to find a poor substrate for LTP. It is also suggested that exogenous lipid particles administered into the circulation may be changed their lipid composition in the same way and then affinities for various apolipoproteins may also be changed (Arimoto et al., 1998; Saito et al., 1996) after administration. It is known that some apolipoproteins bind exogenous lipid particles in a particle size-dependent manner (Morita et al., 2003; Yamamoto et al., 2003) and PLTP also reduces transfer activity with increase in liposome size and decrease in bilayer curvature (Sweeny and Jonas, 1985). Further detailed studies on lipid transfer using different types of lipid–particulate systems, as a donor or an acceptor, are essential for better understanding to achieve successful drug delivery.

4. Conclusion

A nanometer-sized lipid particle, LNS® (25–50 nm in diameter) is a promising new formulation to improve the efficacy of pharmacologically active agents. However, the plasma half-life of various lipophilic compounds administered with LNS and lipids composing LNS particles was markedly different. A major reason for compounds with insufficient lipophilicity may be the release of these compounds from LNS and followed by their association with serum proteins. However, even in a highly lipophilic compound, results in this study suggested that LTP in serum removed compounds from exogenous LNS particles and forwarded them to plasma lipoproteins. Only the lipophilicity of a drug was not a key factor for incorporating and maintaining it in the lipid particles in the blood circulation. We showed that LTP plays a possible key role in the efficacy of drug delivery using lipid emulsion particles. Thus, lower affinity not only to serum albumin but also to LTP seems to be a key to success for derivation of drugs suitable for a lipid-based drug carrier system. The function and substrate specificity of LTP in drug delivery using lipid based particulate system should be investigated in more detail.

Acknowledgements

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