Effect of Serum on Dose- and Temperature-Dependent Hepatic Uptake of Multilamellar Vesicles (MLV)

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Effects of the addition of serum to the perfusate on hepatic uptake of multilamellar vesicles (MLV) were examined in recirculating perfused rat liver. MLV was labelled with both membrane lipid marker ([14C]cholesterol oleate) and aqueous phase marker ([3H]insulin or [5-6]-carboxyfluorescein (CF)). The uptake rates of [14C]cholesterol oleate and CF at 37 °C coincided well, indicating both markers to be taken up as MLV. Insulin was released from MLV at 37 °C but its uptake rate tended to approach that of [14C]cholesterol oleate at 4 °C or at high MLV dose. Some factor in serum promoted MLV uptake at 37 °C, but its effect was inhibited at 4 °C. The promoting effect was indicated to be possibly due to activation of adsorption of MLV to the hepatic Kupffer cell surface and/or that of phagocytosis by the cells. The saturation of MLV uptake was observed with increase in MLV dose, suggesting the saturation of MLV adsorption to the hepatic cell or the consumption of serum factor which promotes MLV uptake.

Keywords — serum factor; multilamellar vesicle; hepatic uptake; dose dependence; temperature dependence; Kupffer cell

Introduction

In order to use liposomes as a carrier in a drug delivery system, the physiological factors influencing the biological fates of liposomes as well as their physicochemical characteristics should be examined in detail. Liposomes administered intravenously are generally taken up by the organs with reticuloendothelial systems (RES), especially by the liver, and eliminated rapidly from the blood.1-3 Such uptake is convenient if the organ with RES is a target one, but inconvenient if the sustained release or targeting to other organs is the objective for the use of liposomes. Accordingly, the appropriate control of the uptake of liposomes to the organs with RES is an important problem for the use as an effective carrier.

Liposomes are well taken up by Kupffer cells whether the liposomal membrane is modified to have the targeting specificities to the parenchymal cells or not.4-6 This phenomenon is induced by the nonspecific physicochemical characteristics of liposomes7-9 or mediated by specific receptors through the opsonization by interaction of liposomes and blood components.10-14

Kiwada et al. examined hepatic uptake of liposomes (multilamellar vesicles (MLV)) from the uptake profile of its membrane lipid marker and found the uptake to be activated by a serum factor.10 They found the serum factor to be a thermolabile protein of with a molecular weight of less than 17000 Da and the interaction of the factor with MLV to be very rapid and irreversible. The purpose of the present study is to clarify whether MLV is taken up into the liver as it is in the presence of serum and which uptake process the serum factor activates. MLV was labelled with both membrane lipid marker and aqueous phase marker. The serum effect on the MLV uptake processes was examined from dose and temperature dependences of uptake rates of both markers.

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Materials and Methods

Materials — Hydrogenated egg phosphatidylcholine was kindly supplied by Nippon Fine Chemicals Co., Ltd. (Osaka, Japan). Dicetylphosphate and cholesterol were obtained from Sigma Chemical Co. (St. Louis, U.S.A.) and Wako Pure Chemical Co., Ltd. (Osaka, Japan), respectively. [Olate-1-14C]cholesteryl oleate ([14C]cholesteryl oleate) and [3H]inulin were purchased from New England Nuclear Co. (Boston, U.S.A.). 5(6)-Carboxyfluorescein (CF) was obtained from Eastman Kodak Co. (Rochester, U.S.A.).

Preparation of MLV — The detailed method followed the reports of Kiwada et al. 15,16 MLV composed of hydrogenated egg phosphatidylcholine, dicetylphosphate and cholesterol (4:1:4 in molar ratio) was prepared by the vortexing method. The size was adjusted by extrusion through a polycarbonate membrane with a pore size of 0.4 μm. [14C]Cholesteryl oleate (1.5 μCi/ml) as a MLV membrane lipid marker and [3H]inulin (50 μCi/ml) as an aqueous phase marker were used. MLV which included 100 mM CF as an aqueous phase marker but no radioactive membrane lipid marker was also prepared for the examination of MLV stability. The marker compounds which were not taken up into MLV were removed by equilibrium dialysis to isotonic phosphate buffer solution (pH 7.4) using the above polycarbonate membrane.

Uptake of MLV by Perfused Rat Liver — The liver of a Wistar male rat (body weight, 300 ± 20 g) was perfused in situ by a small volume circulating system according to the report of Kiwada et al. 15 As the perfused solution, 23 ml of Krebs–Henseleit bicarbonate buffer solution (pH 7.4), which is composed of 118.5 mM NaCl, 4.75 mM KCl, 2.54 mM CaCl2, 1.19 mM KH2PO4, 2.43 mM MgSO4 and 25.0 mM NaHCO3, were used. The solution was saturated with O2–CO2 (95:5) and perfused from the portal vein to the inferior vena cava at 37 °C. For the examination of serum effect, 5.4 ml of fresh rat serum were added to the perfused solution. To examine the dose-dependent uptake of MLV into the liver, MLV including the total lipid amount of 36.0 μmol (high dose), 7.20 μmol (middle dose) and 3.60 μmol (low dose) were added to the above perfusate. In other perfusion experiment, the middle dose was used. During the perfusion for 12 min, radioactivities of [14C]cholesteryl oleate and [3H]inulin in the perfusate were measured at some intervals and their elimination was evaluated by the ratio of remaining radioactivity in the perfusate to the initial one before the perfusion. The remaining amount of CF in MLV was obtained from the difference between the total CF amount in the perfusate and the released CF amount from MLV to the perfusate, and expressed as the ratio to the initially entrapped amount in MLV. The total CF amount in the perfusate and initially entrapped CF amount were determined after MLV was completely disintegrated by mixing the perfusate sample with 5% Triton X-100 aqueous solution of the equal volume. The released CF amount from MLV was determined from the fluorescence intensity of the resultant perfusate after the dilution with purified water since CF entrapped in MLV does not emit fluorescence. The hepatic MLV uptake at 25 and 4 °C were also determined and compared with that at 37 °C.

Stability of MLV in Situ and in Vitro — The stability of MLV during in situ liver perfusion at 37 °C was examined by determining the release of CF from MLV to the perfusate in the presence and the absence of serum. The stability was also determined in vitro by incubation of the same solution as that for the liver perfusion at 37 °C. The released CF amount from MLV was expressed as the ratio to the initially entrapped one.

Assay — The radioactivities of [14C]cholesteryl oleate and [3H]inulin were measured in a liquid scintillation counter (Aloka 903, Tokyo, Japan) after the treatment of the perfusate sample according to the reports of Kiwada et al. 15 The fluorescence of CF released from MLV was measured at 495 nm for the excitation and at 520 nm for the emission after the samples were diluted four times with purified water.

Results

Serum Effect
The effects of serum on the MLV uptake into the liver at 37 °C were examined. Figures 1a and b show the elimination of membrane lipid marker ([14C]cholesterol oleate) and that of aqueous phase marker ([3H]inulin) from the perfusate, respectively. Serum increased the elimination rate of [14C]cholesterol oleate as shown by Kiwada et al.16,17) The elimination rate of [3H]inulin, however, was smaller than that of [14C]cholesterol oleate and not changed by serum. To the contrary, the elimination of CF as another aqueous phase marker was promoted by serum as shown in Fig. 2. The elimination rate of [14C]cholesterol oleate coincided well with that of CF (Figs. 1a and 2). The release ratios of CF from MLV during the in situ liver perfusion and in vitro were less than 5% for 12 min at 37 °C and the addition of serum little affected them (data not shown).

**Temperature Dependence**

The hepatic MLV uptake in the presence of serum was also examined at 4 and 25 °C. The
result at 25 °C was not significantly different from that at 37 °C (data not shown). The time courses of elimination of [14C]cholesterol oleate and [3H]inulin from the perfusate at 4 °C are shown together with that at 37 °C in Fig. 3. Although the elimination rate of [14C]cholesterol oleate at 4 °C significantly decreased in comparison with that at 37 °C, that of [3H]inulin showed a tendency to be promoted by reducing temperature. Thus, the elimination curves of both markers were obviously different from each other at 37 °C but approached closer at 4 °C.

Dose Dependence

Hepatic MLV uptake was examined at three doses, 36.0 μmol (high), 7.20 μmol (middle) and 3.60 μmol (low). Fig. 4a and b show the elimination of [14C]cholesterol oleate and [3H]inulin from the perfusate in the presence of serum, respectively. The dose-dependent elimination of [14C]cholesterol oleate was observed in the presence of serum (Fig. 4a) but not in the absence of serum (data not shown). Although the remaining ratios at the low and middle doses decreased with time, that at the high dose reached the constant level 5 min after the start of perfusion (Fig. 4a). On the other hand, the elimination rates of [3H]inulin was small at all three doses and the dose dependence was slightly observed (Fig. 4b).

The time course of the ratio of [3H]inulin to [14C]cholesterol oleate remaining in the perfusate was shown at each MLV dose in Fig. 5. Although the ratios at the middle and low doses increased with time, that at the high dose was kept approximately one, indicating the elimination patterns of both markers to be almost the same at the high dose.

Discussion

The hepatic uptake of MLV determined with the membrane lipid marker ([14C]cholesterol oleate) was promoted by the addition of serum to the perfusate (Fig. 1a), as reported by Kiwada et al. 15 The serum effect on elimination of the
aqueous phase marker of MLV from the perfusate was found for CF but not for \[^{3}H\]inulin (Figs. 1b and 2). The elimination rate of \[^{3}H\]inulin was smaller than that of \[^{14}C\]cholesterol oleate or CF. When the aqueous phase marker which is water soluble and poorly lipophilic is released from MLV, it is not taken up into the liver by itself. The result that elimination of \[^{3}H\]inulin was slower than that of \[^{14}C\]cholesterol oleate (Fig. 1a and b) was probably caused by the release of inulin from MLV to the perfusate. Since the elimination of CF agreed fairly well with that of \[^{14}C\]cholesterol oleate (Figs. 1a and 2) and the release of CF from MLV was very small both in situ and in vitro, CF is regarded as a more stable marker than \[^{3}H\]inulin. However, CF cannot be used in coexistence with \[^{14}C\]cholesterol oleate since the fluorescence emitted by CF disturbs the measurement of radioactivity. Thus, \[^{3}H\]inulin was used as an aqueous marker of MLV and its release from MLV was further examined.

MLV with the particle size of 0.4 μm in this study is generally taken up by Kupffer cells and its uptake processes are composed of the adsorption (binding) of MLV to the cell surface and the following phagocytic internalization.\(^{18}\) The aqueous phase marker of MLV can be released through interaction of MLV and serum component such as lipoprotein or membrane lipid exchange.\(^{19,20}\) The low perfusion temperature suppresses phagocytosis as shown in the elimination of \[^{14}C\]cholesterol oleate (square symbols in Fig. 3) and/or the release of the aqueous phase marker from MLV. At 4 °C, the elimination of \[^{3}H\]inulin tended to be greater than that at 37 °C (circles symbols in Fig. 3). This temperature-dependent result is considered due to the restriction of \[^{3}H\]inulin release from MLV rather than the increase in the hepatic uptake at 4 °C. The similar elimination patterns of \[^{14}C\]cholesterol oleate and \[^{3}H\]inulin at 4 °C in Fig. 3 supported the above consideration on the temperature-sensitive MLV uptake mechanism. The elimination of \[^{14}C\]cholesterol oleate in the presence of serum decreased with the increase in MLV dose (Fig. 4a). The dose-dependent MLV uptake obtained from the elimination of membrane lipid marker is probably due to saturable adsorption of MLV on the cell surface and/or consumption of the serum promoting factor at the high MLV dose. The elimination of \[^{3}H\]inulin showed no dose dependence (Fig. 4b). It is considered that the ratios of both hepatic uptake of \[^{3}H\]inulin and its release from MLV were reduced with the increase in MLV dose and thus the dose-dependent elimination apparently disappeared. This consideration was supported by the constant ratio of \[^{3}H]/[^{14}C\] as one which attained 5 min after the perfusion at the high dose (Fig. 5), suggesting MLV including two markers to be taken up into the liver cells intact.

In summary, \[^{14}C\]cholesterol oleate and CF are taken up into the liver as MLV at 37 °C. \[^{3}H\]inulin was released from MLV at 37 °C but was taken up as MLV at 4 °C or at a high MLV dose. The serum factor may enhance hepatic MLV uptake by promotion of the MLV adsorption to the Kupffer cell surface and/or activation of the phagocytosis. The saturation of MLV uptake at the high MLV dose can be explained by the saturable adsorption to the cell surface and/or consumption of serum factor. Thus, the most important process in the MLV uptake is the interaction of MLV and Kupffer cells in the presence of serum factor and it is currently under investigation using primarily cultured Kupffer cells.

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