Poly(ethylene glycol) Derivative of Cholesterol Reduces Binding Step of Liposome Uptake by Murine Macrophage-like Cell Line J774 and Human Hepatoma Cell Line HepG2

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Liposome uptake by HepG2 human hepatoma cells was investigated in comparison with the uptake by J774 murine macrophage-like cells. HepG2 cells accumulated liposomes (egg yolk phosphatidylcholine (EPC)/Chol; 75/25, diameter 0.2 μm) at 37 °C comparably to J774 macrophage-like cells. Confocal microscopic observations revealed that J774 cells internalized EPC/Chol liposomes efficiently but HepG2 cells kept most of the liposomes bound on their plasma membrane surfaces. Poly(ethylene glycol) (PEG)-coated liposomes (0.2 μm) containing poly(ethylene glycol) cholesteryl ether (PEG-Chol) avoided cellular uptake at 37 °C by either cell line. In both cell lines, binding of PEG-coated liposomes was lower than that of EPC/Chol liposomes when incubation was carried out at 4 °C. To analyze the binding process at 37 °C, surface-bound liposomes were removed from the cells by pronase treatment. A reduction of the amount of bound-liposomes on cell surfaces was observed in the case of PEG-coated liposomes. Therefore, PEG-coating reduces direct binding of liposomes to the cell surfaces. The presence of apolipoprotein E (apoE) increased the uptake of EPC/Chol liposomes *via* its receptor in both cell lines. In contrast, cellular uptake of PEG-coated liposomes was not enhanced by treatment with apoE. Therefore, while apoE-mediated liposome uptake occurs in the case of EPC/Chol liposomes, it does not occur for PEG-coated liposomes; PEG-coating also inhibits protein-mediated binding to the cells. These results further imply that elusion from liver clearance of PEG-coated liposomes is not only due to the reduction of uptake by Kupffer cells but also by hepatocytes when liposomes are small enough to go through the fenestrates of the endothelial lining.

Key words liposome; poly(ethylene glycol); HepG2; binding; apolipoprotein E; drug delivery system

Phospholipid vesicles (liposomes) have been studied as drug carriers in the last two decades.[3)] After intravenous administration, these liposomes are cleared from the bloodstream rapidly and are accumulated in liver and spleen (reticuloendothelial system, RES).[3)] This phenomenon becomes an obstacle for drug delivery to other cells of the body. Some blood proteins (opsonins) are thought to coat the liposomes and transform them into a recognizable entity for RES,[4)] but *in vitro* experiments with phagocytic cells revealed that liposomes were also taken up without opsonization.[5)] Moreover, it has been shown that some liposome binding sites are present on the cell surface that are opsonin-independent.[6)] Therefore, cells could take up liposomes through direct recognition in addition to an opsonin-mediated one.

Reducing the high level accumulation of liposomes to RES has been achieved by surface modification of liposomes, *i.e.*, using amphiphilic poly(ethylene glycol) (PEG) derivatives incorporated into liposome membranes.[7)] PEG-coated liposomes showed a longer circulating time in blood and lower accumulation into RES than non-coated, conventional liposomes. So far, the most common derivatives used to prepare the PEG-coated liposomes have been phosphatidylethanolamine conjugates.[7)] However, liposomes coated with cholesterol (Chol) derivatives also exhibited longer circulation time in blood and less accumulation to RES than conventional liposomes.[8)] It has been proposed that the large steric hindrance and/or hydrophilic barrier caused by the PEG moiety could prevent liposomes from being opsonized by serum proteins.[9)] PEG-coating has been suggested to reduce the direct interaction of liposomes with the cell surfaces as well as opsonin-mediated recognition.

In vivo, the liver provides the largest trapping sites for liposomes. Recent studies have pointed out the involvement of non-phagocytoic parenchymal cells in the uptake of the administrated liposomes. Scherphof and collaborators reported that inhibition of uptake of liposomes by PEG modification was far more pronounced for the hepatocytes than for the Kupffer cells.[10)] It was also shown that on liver cryosections, PEG-coated liposomes were specifically located into the Kupffer cells and not found in parenchymal cells.[11)] These results suggested liposome uptake by parenchymal cells is more sensitive to PEG modification than that by macrophages.

We have reported the effect of PEG modification on liposome uptake by J774 macrophage-like cells using different PEG derivatives of cholesterol.[12)] In this study, we investigated the celluar uptake of liposomes by hepatoma cell line HepG2 as a model of parenchymal cells. Treating the cells, both J774 and HepG2 cells, with pronase, we estimated the liposome binding on cell surfaces and internalizaton by phagocytosis and/or other unknown routes. Moreover, the PEG modification effect on liposome binding and internalization was investigated by pronase treatment and the effect on protein-mediated interaction between liposomes and cells was also investigated using apolipoprotein E3.

Materials and Methods

Materials Egg yolk phosphatidylcholine (EPC) was kindly provided by Asahi Kasei (Tokyo, Japan). Chol was supplied by Wako Pure Chemical (Osaka, Japan) and was used after double recrystallization from ethanol. Poly(ethylene glycol) cholesteryl ether (PEG-Chol) with an average

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number of ethylene glycol units of 100 and 200 which have an average molecular weight of about 4400 and 8800, respectively, were synthesized by Nikko Chemical (Tokyo, Japan).[8] *N*-(monoethoxy poly(ethylene glycol) succinyl) phosphatidylethanolamine (PEG-PE) of PEG Mr 5000 containing 114 ethylene glycol units was kindly provided by Therumo Pharmaceutical (Osaka, Japan). All other chemicals from Wako Pure Chemical were of special grade. Water was purified by double distillation in a glass still.

Liposome Preparation Liposomes were prepared in Hepes buffer (NaCl; 137 mM, KCl; 6 mM, Hepes; 10 mM; pH 7.4) by the extrusion method through 0.2 μm of polycarbonate filter (Nuclepore, Coster, U.S.A.) as previously described.[8,12] Lipid compositions of control liposome, 5 and 25 mol% PEG-liposomes are described in Table 1. Labeling the liposome membranes was performed using either 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) or *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) added to the appropriate lipid mixtures before solvent evaporation. Phospholipid concentration was assayed by the Bartlett method.[13] The average size of liposomes, measured by dynamic light scattering using a laser particle analyzer LPA-3100 (Ostuka Electronics, Tokyo, Japan), is described in Table 1. No significant difference could be observed in function of the PEG length.

Determination of Liposomes Lamellarity The lamellarity of liposomes was checked by using the dithionite assay.[14] Briefly, 2.0 ml of liposomes symmetrically labeled with NBD-PE (0.25 mol%) were incubated at 30 °C in a cuvette. The 25 mol% PEG(200)-liposome was assayed after gel-filtration on a Sepharose CL-4B column equilibrated by Hepes buffer to remove PEG-Chol micelles that contained part of NBD-PEs. The monitoring of NBD fluorescence was started before the addition of 20 μl of 1 M sodium dithionite/1 M Tris to the sample and stopped after all NBD fluorescence was quenched by the addition of 20 μl of 10% Triton X-100. The fluorescence intensity was monitored on a spectrofluorometer RF 5000 (Shimadzu, Kyoto, Japan) whose cuvette holder was thermostated at 30±0.5 °C. The excitation and emission wavelengths were 450 and 530 nm, respectively. Fluorescence intensity was normalized to the intensity before dithionite addition. Values used for the calculation of lamellarity of vesicles and number of vesicles constituted from mol lipid as follows: bilayer thickness, 4 nm[15]; bilayer distance, 3.8 nm for control liposome and 23 nm for PEG-coated liposomes which were determined from electron cryomicroscopic observation and corresponded to the X-ray diffraction data presented by Kenworthy and collaborators[16]; molecular area, 74 Å^2 for EPC,[17] 41 Å^2 for Chol[18] and 41 Å^2 for PEG-Chol which was assumed to be the same as Chol, although the molecular area of PEG-Chol determined from the Gibbs equation was larger than that of Chol.[8]

Cell Culture and Cell Preparation A murine peritoneal macrophage-like cell line J774, a human hepatoma cell line HepG2 and a human fibrosarcoma cell line HT-1080 were grown in monolayer using Dulbecco's Modified Eagle Medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS; Bio Whittaker, Walkersville, MD, U.S.A.), L-glutamine, penicillin and streptomycin (Cosmo Bio, Tokyo, Japan). The cells in culture flasks were scraped off by trypsin (0.025%) treatment for HepG2 cells and HT-1080 cells or EDTA (0.2%) treatment for J774 cells (10 min at 37 °C), centrifuged, rinsed and counted. Cells (1.5×10^5) were then plated in 6-well plates or culture dishes (Nunc for J774 and HepG2, Falcon for HT-1080), allowed to recover in a CO_2 incubator at 37 °C for 2 d and used in the following experiments unless mentioned otherwise.

Cellular Uptake of Liposomes DiI-labeled liposomes (0.4 mol%) were preincubated for 10 min at 37 °C in DMEM 10% FBS. Cells, plated at 1.5×10^5 per dish 2 d before, were incubated with the liposomes at the desired concentration and period in 10% FBS at 37 °C in a humidified incubator or at 4 °C. At intervals, cells were washed with ice-cold Hepes buffer 3 times and dissolved in Hepes buffer containing 0.2% Triton X-100. After centrifugation (10000×***g***, 10 min, 4 °C), supernatant was assayed with DiI fluorescence with excitation and emission wavelengths at 550 and 565 nm, respectively, using a spectrofluorometer RF 5000 (Shimadzu, Kyoto, Japan). When the results were represented as nmol of lipid associated to 10^6 cells, the amount of cellular protein was assessed by the method of Bradford[19] using human transferrin as a standard. Protein amount contained in 10^6 cells was 0.158, 0.163 and 0.233 mg, for J774, HepG2 and HT-1080, respectively.

When the effects of apolipoprotein E (apoE) on liposome uptake by the cells were investigated, liposomes (1.5 mM) were pre-treated with apoE (7.5 μg/ml) by incubation for 30 min at 37 °C. Then, they were diluted in serum free Hepes DMEM to a liposome concentration of 100 μM lipids. Cells were kept in serum free Hepes DMEM for 1 h in a humidified chamber in a shaking incubator at 37 °C and then incubated with apoE-treated liposomes at 100 μM. The same procedure was applied using bovine serum albumin (BSA, 7.5 μg/ml) instead of apoE. In competitive inhibition experiments, the cells were incubated in medium containing both apoE treated liposomes and lipoproteins from bovine plasma (Sigma, St. Louis, MO) at 78 μg/ml, equivalent to the amount presented in medium containing 10% FBS.[20]

Pronase treatment was performed to separate surface-bound liposomes from the cells, in order to analyze the binding and internalization process of liposomes by the cells incubated at 37 °C. The cells, plated at 1.5×10^5 per dish 2 d before, were incubated with DiI-labeled liposomes at 100 μM in Hepes DMEM 10% FBS in a shaking incubator at 37 °C. After incubation with liposomes, cells were washed with ice-cold Hepes buffer 5 times and then incubated with 0.1% pronase in Hepes buffer at 4 °C. After the cells were detached from the dishes, the cell suspension was collected in Eppendorf tubes which contained 30% sucrose solution. Tubes were then centrifuged at 10000×***g*** for 10 min at 4 °C. The supernatant was collected as the fraction containing surface-bound liposomes. The pellet of cells was dissolved in 0.2% Triton X-100 and the lysate was centrifuged at 10000×***g*** for 10 min at 4 °C. The supernatant of the cell lysate was collected as the fraction containing internalized liposomes. The associated fluorescence of DiI in each fraction was quantified. The involvement of phagocytosis in liposome internalization was investigated using cytochalasin treatment to inhibit the actin-dependent internalization by cells. Cytochalasin D (CytoD) dissolved in dimethyl sulfoxide

(DMSO) was diluted in medium to a concentration of 1 μg/ml. The cells were preincubated for 30 min at 37 °C in medium containing CytoD and then incubated with liposomes in the presence of CytoD.

Confocal Microscopic Observation The association of DiI-labeled control liposomes was visualized under confocal microscopy. Cells (*ca.* 2×10^4 cells) on a coverslip were incubated with control liposomes in a humidified chamber at 30 °C for 2 h. After washing, the cells were observed in buffer by mounting the coverslip on a slide glass using a piece of parafilm as spacer. The cells were observed under a confocal laser scanning system (MRC-600, BioRad, Hercules, CA) coupled with an Axiophoto microscope (Carl Zeiss, Germany) at 25 °C.

Results

Lamellarity of Liposomes We studied the interaction of various liposomes with cells by quantifying the cell-bound liposomal lipid or fluorescence. In order to calculate the number of liposomes, we determined their lamellarity by using NBD-PE in combination with dithionite. Generally, liposomes composed of EPC and Chol extruded through 0.2 μm filter were thought to be multilamellar. The PEG chain from each bilayer expanded into the space between the bilayers.[16] Therefore, the lamellarity of PEG-coated liposomes might be different from that of control liposomes composed of EPC and Chol (75/25). When unilamellar vesicles homogeneously labeled with NBD-lipids were in contact with dithionite, only NBD-lipids located in the outer leaflet of the liposome bilayers were irreversibly quenched.[14] In the case of pure unilamellar vesicles of a 200 nm diameter, the fluorescence should be quenched by 51%.

Figure 1 shows the kinetics of reduction of NBD-fluorescence by dithionite. The liposomes were incubated at 30 °C while the fluorescence intensity at 530 nm was monitored. The addition of dithionite at time 0 selectively reduced the NBD groups exposed to the external aqueous phase. The initial value of fluorescence intensity of NBD in the control liposome was reduced by 34% within 2 min (upper trace). The bottom trace shows the data obtained with 5 mol% PEG (200)-liposome. Traces recorded for 5 and 25 mol% PEG (200)-liposomes were not distinguishable. Both types of PEG-coated liposomes showed a 49% reduction from the initial value. None of the liposomes showed a slow decrease of NBD fluorescence on a longer time scale, indicating that liposomes were not permeable to dithionite, making impossible the quenching of inner monolayer-halves or 2nd bilayers. The suspension of 5 mol% PEG-liposome did not contain PEG-Chol micelles, while a few percent were present in the 25 mol% PEG-liposome suspension.[8] Therefore, PEG-liposomes were examined after gel-filtration through Sepharose 4B column. The micelles contained 9% of the total NBD-PE added (not shown). Addition of Triton X-100 decreased the NBD fluorescence to zero, as dithionite reduced all chromophores present in the cuvette. The results are summarized in Table 1; we estimated the lamellarity of liposomes and the number of vesicles formed in one mol lipid, from the ratio of NBD-PE distributed in outermost leaflet of liposomes. All PEG-coated liposomes had a smaller number of lamellae than control liposome. It had been shown that EPC liposomes, sized with 0.2 μm filter, had 30—35% of total lipids

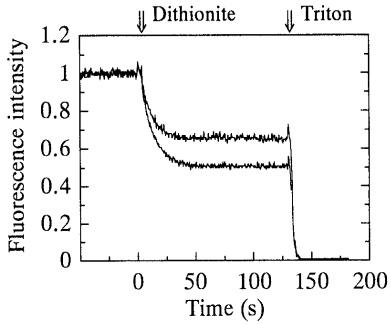

Fig. 1. Kinetics of Reduction of Symmetrically Distributed NBD-PE by Dithionite

Dithionite was added at time 0 to liposomes (100—140 μM) symmetrically labeled with 0.25% NBD-PE. The addition of 10% Triton X-100 (20 μl) made all NBD lipids accessible to dithionite. The upper trace shows the typical data of control liposomes (EPC/Chol; 75/25) and the bottom trace shows the data of 5 mol% PEG-liposomes (EPC/Chol/PEG(200)-Chol; 75/20/5). Traces of 25 mol% PEG-liposomes (EPC/PEG(200)-Chol; 75/25) were not distinguishable from that of 5 mol% PEG(200)-liposomes.

Table 1. Characteristics of Liposomes

Liposomes	Control liposome	5 mol% PEG(200) liposome	25 mol% PEG(200) liposome
Composition (EPC/Chol/PEG(200)-Chol)	75/25/0	75/20/5	75/0/25
Diameter[a] (nm)	218.2±10.3	185.1±8.6	178.4±8.7
Outermost leaflet[b] (% of total lipid)	34.2±0.4	48.2±0.5	49.0±0.2
Lamellarity[c]	1.5	1.1	1.1
Number of vesicles per mol lipid[c]	9.0×10^{17}	17.7×10^{17}	17.9×10^{17}

a) Diameters of liposomes were determined by dynamic light scattering. *b*) Percentage of lipid in outermost leaflet was determined by the dithionite assay. *c*) Lamellarity and vesicle number in mol lipid were calculated from values in this table and text.

in their outermost monolayer, which was quantified by reduction in the ^{31}P-NMR signal intensity by the addition of external Mn^{2+}.[21] The authors have also observed 3 or more lamellae per multilayer vesicles by occasional cross-fractures of freeze-fracture microscopy and suggested at least 90% of the vesicles were unilamellar.[21] These results are in agreement with our present estimation with control liosomes. Finally, the number of vesicles of the two types of PEG-coated liposomes were similar but were twice as many as that of control liposomes.

Comparative Cellular Uptake of Liposomes We investigated the liposome uptake by hepatocytes and the effect of PEG modification, using HepG2 cells in comparison with other cell lines. Figure 2 shows control liposome (EPC/Chol; 75/25) association to human hepatoma cell line HepG2 compared with macrophage-like J774 cells and human fibrosarcoma cell line HT-1080, after 2 h incubation. J774 cells took up liposomes efficiently after incubation at 37 °C and less at 4 °C, as described previously.[12] The associated amount obtained at 37 °C, (internalized and remaining surface-bound) was larger than that at 4 °C indicating that J774 cells actively phagocytosed liposomes bound on their surfaces. Approximately 420 vesicles were associated to one J774 cell after incubation at 37 °C for 2 h with control liposomes 100 μM. In the case of HepG2 cells, the associated amount at 37 °C was similar to that in J774 cells but the binding amount at 4 °C

was larger. Because the difference in the associated amount between 37 and 4 °C was smaller than that of J774 cells, HepG2 cells seemed to internalize liposomes less effectively than J774 cells. However, HT-1080 cells, a fibrosarcoma cell line, did not show significant association of liposomes after incubation at 37 or 4 °C.

Cells were observed under confocal microscopy after 2 h incubation at 30 °C with control liposomes at 100 μM labeled with DiI. J774 cells had strong fluorescent spots inside the cells as can be seen in the picture of the medium plane presented in Fig. 3A. However, HepG2 cells, shown in Fig. 3B on a projection of 12 planes, had fluorescent spots aligned to form rings, which appeared to be at the plasma membrane level, and a few fluorescent spots inside. These micrographs indicated that J774 macrophage-like cells internalized liposomes efficiently but in HepG2 cells, liposomes were not internalized efficiently or were not transported centripetaly after normal internalization.

Effect of PEG Modification on Liposome Uptake PEG-coated liposomes were prepared with PEG-Chol containing 100 and 200 ethylene glycol units, respectively, or PEG-PE containing 114 ethylene glycol. *In vivo*, PEG-Chol liposomes showed a reduced accumulation to liver and an increased circulating amount in blood 8 h post injection.[8]

Figure 4A shows the effect of PEG modification on liposome uptake at 37 °C by J774 and HepG2 cells. To allow a comparison between the two cell lines, results are expressed in percent of control liposomes, *e.g.*, 138.3±13.3 pmol lipid per dish for J774 cells and 268.3±29.9 for HepG2 cells for liposomes at 100 μM. The presence of PEG-coating on liposomes induced a decrease in the associated lipid amount to either cell line. The decrease of association observed for 5 mol% PEG-Chol liposomes was more apparent with HepG2 hepatoma cells than with J774 macrophage-like cells. Similarly, liposomes prepared with 25 mol% PEG-Chol that contain membrane bound molecules at 14 or 16 mol% for PEG(100) or PEG(200)-Chol, respectively[8] and those containing PEG-PE showed also less association to HepG2 cells. The effect of PEG-Chol on liposome uptake was stronger than that of PEG-PE. The reduced association of liposomes containing 5 mol% PEG derivatives seem to be due only to the PEG coating on liposome surfaces while the decrease observed with 25 mol% PEG-liposomes was owing to both PEG coating on the liposome surfaces and free PEG-Chol molecules in the incubation medium.

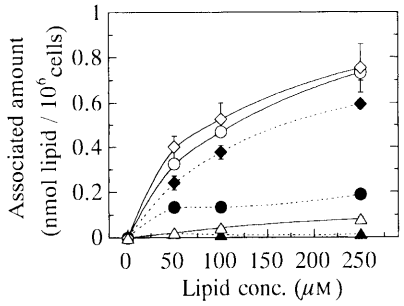

Fig. 2. Liposome Association as a Function of Lipid Concentration

J774 cells (circle), HepG2 cell (diamond) and HT-1080 cells (triangle) were incubated with control liposomes (EPC/Chol; 75/25) at 100 μM in presence of 10% FBS for 2 h at 4 °C (filled symbol) and 37 °C in a CO_2 incubator (open symbol). Results are expressed in nmol lipid/10^6 cells; associated lipid amount was determined from fluorescence intensities of DiI liposome marker and cell number was determined from protein amount assayed by the Bradford method.[19] Data represent mean±S.E. for at least two separate experiments duplicated.

The amount of associated liposomes to the cells was decreased by PEG modification. However, due to the higher lamellarity, the number of vesicles per unit amount of lipid is smaller for the control liposomes. Nevertheless, even after such reassessement, PEG modification still affected cellular uptake. When cells were incubated, 2 h at 37 °C, with liposomes at 9×10^{10} vesicles/ml (corresponding to 100 or 50 μM for control or PEG-coated liposomes, respectively) the number of control liposome associated with J774 or HepG2 cells were $12.5\pm2.1\times10^7$ vesicles/dish or $24.1\pm2.7\times10^7$ vesicles/dish, respectively. However, in the same conditions but in the presence of PEG-liposomes, the associated vesicle number of PEG(200)-liposomes was representing less than 50% of control for J774 cells or less than 15% for HepG2 $4.4\pm0.9\times10^7$ vesicles/dish or $3.1\pm0.5\times10^7$ vesicles/dish, respectively for 25 mol% PEG(200). Similar results were obtained for PEG(100)-Chol (data not shown). The results presented in Fig. 4B showed a reduction of PEG-coated liposome binding to both cell lines at 4 °C after 2 h incubation. Again, to allow a direct comparison between the two cell lines, results were expressed in percent of control liposomes *e.g.* 78.2±7.0 pmol per dish for J774 cells and 109.6±3.9 for HepG2 cells for liposomes at 100 μM.

Binding and Internalization of Liposomes Quantitative analysis of liposome internalization by cells was per-

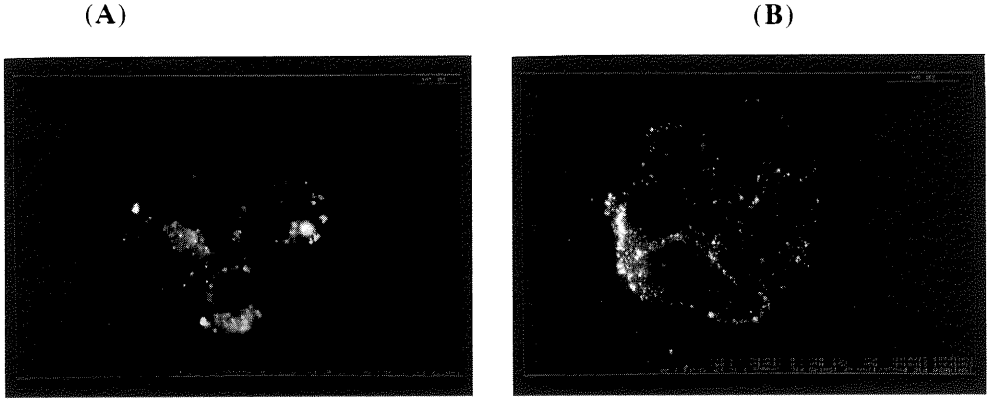

Fig. 3. Typical Confocal Fluorescence Images of the Cells Incubated with DiI Labeled Control Liposomes

J774 cells (A) and HepG2 cells (B) on coverslips (*ca.* 2×10^4 cells) were incubated with control liposomes at 100 μM at 30 °C for 2 h. J774 cells contain DiI fluorescence inside the cells. In contrast, HepG2 cells, visualized by projection, show fluorescence rings, suggesting most of the surface-bound liposomes were not internalized.

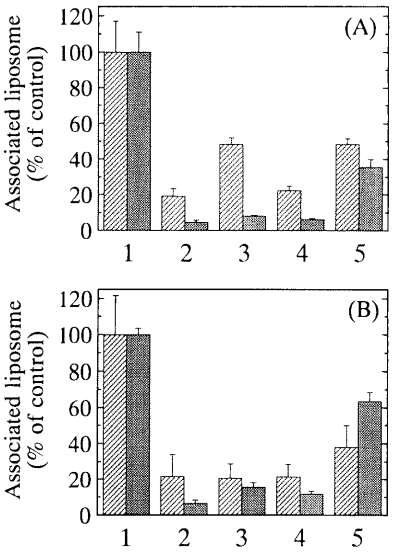

Fig. 4. Effect of PEG Modification on Liposomal Lipid Association to the Cells

Liposomes at 100 μM were incubated with J774 cells (hatched bar) and HepG2 cells (filled bar) for 2 h in presence of 10% FBS at 37 °C (A) and 4 °C (B). Results are expressed as a percentage of associated lipid amount of control liposome to each cell line. 1: control liposome (EPC/Chol; 75/25); 2: 25 mol% PEG(200)-liposome (EPC/PEG(200)-Chol; 75/25); 3: 5 mol% PEG(200)-liposome (EPC/Chol/PEG(200)-Chol; 75/20/5); 4: 25 mol% PEG(100)-liposome (EPC/PEG(100)-Chol; 75/25); 5: PEG-PE (Mr 5000 containing 114 ethylene glycol units) liposome (EPC/Chol/PEG-PE; 50/25/25). Data represent mean±S.E. for at least three separate experiments duplicated.

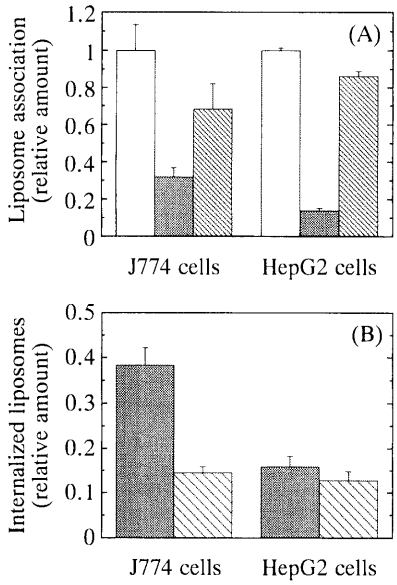

Fig. 5. Separation of Liposomes Associated to Cells by Pronase Treatment

(A): The cells were incubated with control liposomes at 100 μM in the presence of 10% FBS at 37 °C for 2 h. Liposomes bound on the cell surface were separated from internalized liposomes by pronase treatment. Total uptake of liposomes (open bar) was the sum of internalized liposomes (filled bar) and cell surface bound liposomes (hatched bar). (B): Internalized liposome amount was investigated with non-treated cells (filled bar) and cytochalasin D treated cells (hatched bar) after 1 h incubation at 37 °C. Results are expressed relative to the amount of total association to non-treated cells. Data represent mean±S.E. for two separate experiments duplicated.

formed using pronase treatment. After incubation at 37 °C, DiI-labeled liposomes that remained bound to the cell surface was removed by treating the cells with 0.1% pronase at 0 °C for 30 min. The fluorescence from the internalized liposomes was quantified by dissolving cells with 0.2% Triton X-100. DiI fluorescence was completely released from cells when the internalization was inhibited using incubation at 4 °C. To allow a direct comparison between the two cell lines, results were expressed in relative amount of total association of control liposomes to non treated cells, *e.g.*, 503±96 pmol per dish for J774 cells and 1085±211 for HepG2 cells. After 2 h-incubation at 37 °C, J774 cells internalized one third of liposomes associated to the cells while HepG2 cells internalized only one fifth (Fig. 5A). These results indicated that J774 cells internalized liposomes efficiently and HepG2 cells kept most of them at their surface.

Interestingly, when cells were treated with a microfilament-disrupting agent, CytoD at 1 μg/ml, internalization of liposomes by J774 cells was drastically reduced (Fig. 5B). In contrast, CytoD did not change the pronase releasable liposomes fraction in HepG2. These results indicated that the internalization in HepG2 cells is mediated by actin-independent endocytosis and that J774 internalized 62% of total liposomes by phagocytosis.

By using pronase treatment, we also found that binding of liposomes to the cell surface is more efficient at 37 °C than at 4 °C. For example, binding of control liposome incubated for 1 h at 100 μM at 4 °C was 48±10 pmol per dish for J774, while at 37 °C it increase by 3.5-fold to as much as 165±29. The difference was expanded in HepG2 cells amounting 6.5-fold with values 87±11 at 4 °C *versus* 578±113 pmol per dish at 37 °C.

In order to analyze the effect of PEG-coating on the binding step of liposome uptake by the cells at 37 °C, pronase treatment was performed to the cells incubated with PEG-liposome in medium containing 10% FBS at 37 °C for 4 h. As shown in Fig. 6A for J774 cells and Fig. 6B for HepG2 cells, the presence of 5 mol% PEG(100)-Chol induced an large reduction of liposomes binding and internalization. Results

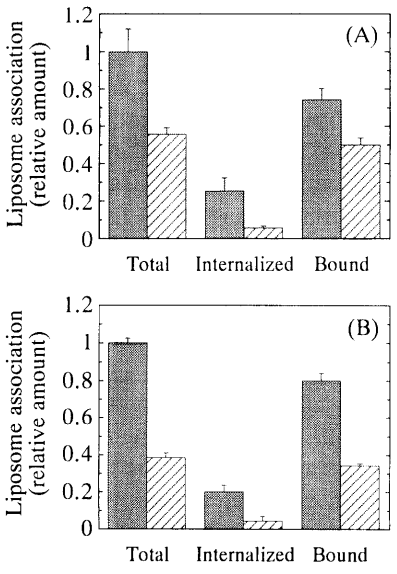

Fig. 6. Effect of PEG Coating on Liposome Binding and Internalization by the Cells

J774 cells (A) and HepG2 cells (B) were incubated with control liposomes (EPC/Chol; 75/25, filled bar) or 5 mol% PEG(100)-liposomes (EPC/Chol/PEG(100)-Chol; 75/20/5, hatched bar) at 100 μM in the presence of 10% FBS at 37 °C for 2 h in a humidified chamber in a shaking incubator. Separation of cell-surface-bound liposomes from internalized liposomes was performed by pronase treatment. Total associated liposomes was the sum of surface-bound and internalized liposomes. Results are expressed relative to the amount of total association of control liposome by each cell line. Data represent mean±S.E. for two separate experiments duplicated.

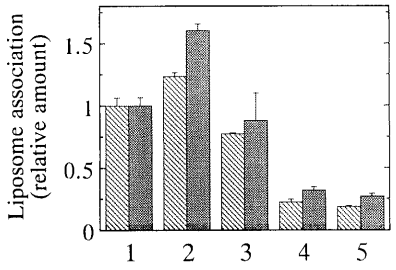

Fig. 7. ApoE Enhancement of Liposome Uptake by the Cells

Control liposomes (EPC/Chol; 75/25) and 5 mol% PEG(200)-liposomes (EPC/Chol/PEG(200)-Chol; 75/20/5) at 1.5 mM were incubated with apoE (7.5 μg/ml) at 37 °C for 30 min before contact with the cells. J774 cells (hatched bar) and HepG2 cells (filled bar) were then incubated with apoE treated liposomes at 100 μM in the absence of serum at 37 °C in a humidified chamber in a shaking incubator for 2 h. 1: control liposome without apoE; 2: control liposome with apoE; 3: control liposome with apoE plus lipoproteins; 4: PEG-liposome without apoE; 5: PEG-liposome with apoE. Results are expressed relative to the associated amount of control liposomes without apoE treatment. Data represent mean±S.E. for two separate experiments triplicated.

were expressed in relative amount of total association of control liposomes to non treated cells *e.g.* 932±113 pmol per dish for J774 cells and 965±244 for HepG2 cells. The reduction of bound liposomes, almost proportional to the reduction of total association 519±31 pmol per dish for J774 cells and 371±25 for HepG2 cells, suggested that the inhibitory effect of PEG coating on cellular uptake was due to an inhibition of liposomes binding on the cell surfaces.

Effect of Apo E on Cellular Uptake of Liposomes Serum protein-mediated interaction between liposomes and cells was investigated using human apoE which mediates lipoprotein uptake by cells through receptor-mediated endocytosis.[22] Liposomes (1.5 mM) were preincubated with apoE at 7.5 μg/ml for 30 min at 37 °C before dilution in serum free DMEM at 100 μM. Aggregation of liposomes was not observed after incubation with apoE (not shown). The cells preincubated in serum free medium for 1 h, were then incubated in medium containing apoE-treated liposomes for 2 h at 37 °C (Fig.7). Results were expressed relative to the amount of total association of non treated control liposomes to cells *e.g.* 5.0±0.6 nmol/mg protein for J774 cells and 3.1±0.4 for HepG2 cells at 37 °C. Uptake of control liposomes was increased by apoE treatment for both J774 and HepG2 cells (bar 2). This enhancement was inhibited by the presence of lipoproteins from bovine plasma in the medium (bar 3). Moreover, when liposomes were treated with BSA instead of apoE, no changes in the liposome association were detected (data not shown). Therefore, the observed enhancement of liposome association should be specifically mediated by apoE binding to the receptors on the cell surfaces. In contrast, cellular uptake of 5 mol% PEG(200)-liposomes (bar 4) was not increased by apoE treatment (bar 5), indicating that the coating liposomes with PEG derivatives inhibited apoE-mediated binding to the cells as well as non-specific one.

Discussion

In this report, we studied the interaction of EPC liposomes with a hepatoma cell line HepG2 and compared the results to that with a macrophage-like cell line J774. The size distribution of the liposomes (180—220 nm) was narrow, 67% of which ranged in ±20 nm. At 37 °C, the amount of cell-associated liposomes was slightly higher in HepG2 cells than in J774 cells. At 4 °C, the difference in the amount of bound-liposomes became larger. All the bound liposomes at 4 °C were released by pronase-treatment in both cells. However, the amount of internalized liposomes that were resistant to pronase-treatment was 2-fold greater in J774 cells compared to HepG2. Liposomes internalization in HepG2 were not affected by CytoD, while, in J774 cells, about 60% was inhibited by this microfilament-disrupting agent. Taking these results together, we can say that the surface of HepG2 cells provides a more efficient binding site for liposomes, while the smaller binding capacity of J774 cells was compensated for by an actin-dependent phagocytosis for clearance of surface-bound liposomes. Macrophages are known to exhibit not only phagocytic but also pinocytic activities including clathrin-dependent adsorptive endocytosis which is not inhibited by CytoD. Our results are in agreement with reports in which the authors using a panel of homogeneous dispersion of polystyrene particles, indicated that macrophages internalize the particles ranging 100 to 1100 nm by both actin-dependent and -independent mechanisms.[23] The scale of this actin-independent mechanisms of J774 cells was similar to that of non-phagocytic HepG2 cells.

The components on the cell surface used for liposomes binding in the presence of serum at 37 or 4 °C are likely protein(s). Early work by Pagano and Takeichi reported that trypsin-treatment of cells removed only 50% of liposomes bound on the surface at 2°C. They incubated cells with phosphatidylcholine (PC) liposomes in the absence of serum at different temperature ranging from 2 to 37°C.[24] They suggested the trypsin-insensitive site to be an accessible region of plasma membrane lipids. Our study indicated that, if incubated in the presence of serum at 4 °C, all the bound liposomes were released by pronase. This result suggests that protease-insensitive binding sites became masked by serum components.

While serum masks the trypsin-insensitive site for liposome binding, certain specific serum proteins promote liposome association. Scherphof and collaborators suggested that apoE and apoA, which mediates lipoprotein uptake by cells, might step in as opsonin for liposome uptake by hepatocytes.[10] In this study, we checked the apoE effect and found that it increased liposome uptake. In blood, apoE exists as bound to lipoproteins. Early work by Bisgaier and collaborators reported that apoE bound to sonicated small PC liposomes and that apoE-bound liposomes were taken up twice as efficiently by HepG2 cells.[25] Our results indicate a similar effect observed for liposomes of larger size. Moreover, this effect was observed with macrophages as well as hepatic cells. Because apoE enhanced liposome uptake 1.6 and 1.2-fold in HepG2 and J774 cells, respectively, internalization of these liposomes are likely promoted by actin-independent mechanisms.

When coated with PEG-Chol, surface-bound liposomes at 4 °C were reduced by 80% in both cell lines (Fig. 4b), suggesting that introduction of a hydrophilic bulky layer at the liposome surface inhibited the interaction of the liposomal core with the cell surface. Such an effect became weaker at 37 °C, where cell-bound liposomes were reduced by 33% in J774 and 57% in HepG2 cells, respectively. This temperature-dependence was likely due to incomplete inhibition of liposomes binding by PEG modification. In contrast to the effect on binding, the fraction of liposomes resistant to

pronase-treatment was reduced by 77% in J774 cells and 78% in HepG2 cells at 37 °C when liposomes were coated with PEG. These results indicate that incorporation of PEG-Chol in liposomal surface inhibited both actin-dependent and -independent cellular activity for internalization of bound liposomes. Since the phagocytic activity in J774 cells is more extensive than the CytoD-insensitive internalization in HepG2 cells, smaller inhibition of the total association of liposomes to HepG2 cells will likely result. However, the reduction of binding by coating liposomes with PEG-Chol observed, *in vitro*, appears sufficient to account for the longer retention of liposomes in the blood stream *in vivo*.[8)]

Moreover, PEG-coating will inhibit ligand-mediated internalization, since apoE-mediated uptake was effectively inhibited by PEG-coating. One interpretation of these results is that binding of PEG-Chol liposome to the cellular protein was not stable enough to provide the anchor for membrane invagination or extension which captured bound liposomes into endocytic/phagocytic vesicles. Another possibility is that inter-membrane exchange of PEG-Chol from the bound-liposomes to the cell surface reduced endocytic/phagocytic cell membrane dynamics. The latter is suggested by reduction of endocytic/phagocytic activity by the PEG-Chol molecules applied in a form of aqueous dispersion in a dose dependent manner.[12)] It is important to note that residual internalization of PEG-Chol liposomes continued to increase during the incubation. Therefore, although both cases are not exclusive, the former mechanism appears to account for a large part of the inhibition. Preliminary experiments show that the amount of apoE bound to control liposomes or PEG-Chol liposomes was very similar indicating that apoE-mediated uptake was inhibited by PEG-coating which sterically hindered the recognition of the apoE receptor (data not shown). Systematic approaches including electron microscopy will be necessary for the elucidation of further details.

In conclusion, Hepatoma cells can take up liposomes from the medium comparably to macrophages, as they provide effective binding sites for liposomes which compensates for the lack of an actin-dependent internalization. PEG-coating on liposome inhibits the binding step of cellular uptake of liposomes, independently of mediation by specific proteins. This effect was observed with both cell lines. These results obtained *in vitro* are qualitatively corresponding to the results of *in vivo* experiments with rats.[8)] Therefore, the improvement of blood circulation time of PEG-coated liposomes is thought to be caused by the inhibition of liposome binding to cells.

References and Notes

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