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The role of β_2-glycoprotein I in liposome–hepatocyte interaction

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Abstract

Adsorption of serum proteins to the liposomal surface plays a critical role in liposome clearance from the blood. The aim of this study was to investigate the role of liposome-adsorbed serum proteins in the interaction of liposomes with hepatocytes. We analyzed the serum proteins adsorbing to the surface of differently composed small unilamellar liposomes during incubation with human or rat serum, and found that one protein, with a molecular weight of around 55 kDa, adsorbed in a large amount to negatively charged liposomes containing phosphatidylserine (PS) or phosphatidylglycerol (PG). The binding was dependent on the liposomal charge density. The ~55-kDa protein was identified as β_2-glycoprotein I (β_2GPI) by Western blotting. Despite the high affinity of β_2GPI for strongly negatively charged liposomes, in vitro uptake and binding experiments with isolated rat hepatocytes, Kupffer cells or liver endothelial cells, and with HepG2 cells showed no enhancing effect of this protein on the association of negatively charged liposomes with any of these cells. On the contrary, an inhibitory effect was observed. We conclude that despite abundant adsorption to negatively charged liposomes, β_2GP1 inhibits, rather than enhances, liposome uptake by liver cells.

Keywords: Liposome; β_2-glycoprotein I; Opsonin; Hepatocyte; Kupffer cell; Liver endothelial cell

1. Introduction

Liposomes are suitable carriers for the selective delivery of drugs to cells in the liver, including hepatocytes and sinusoidal endothelial cells [1–3]. The critical role of the reticuloendothelial system (RES) in the elimination of systemically administered liposomes is well documented, but the involvement of the hepatocytes in this process is scarcely recognized [4]. Previous studies in our laboratory have shown that small uncharged liposomes (<100 nm), which have easy access to hepatocytes by virtue of the open fenestrae in the hepatic sinusoidal endothelial cell lining, are mainly taken up by hepatocytes [3]. The mechanisms involved in the interaction of liposomes with hepatocytes are still largely unclear. It has been reported by many investigators that the adsorption of serum proteins to the liposomal surface plays an important role in the effective clearance of liposomes from the blood [5–8]. Several serum proteins have been proposed to serve as liposomal opsonins, e.g., complement components [9,10], immunoglobulins [8], fibronectin [11,12], α2-macroglobulin [13] and β_2-glycoprotein I (β_2GPI; apolipoprotein H) [14]. Most authors report that the opsonin-like proteins facilitate the uptake of liposomes by macrophages in the liver and spleen. We have, on the other hand, obtained evidence that opsonization may also be required to establish an interaction between liposomes and hepatocytes [4].

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In the present study, we investigated the protein adsorption patterns of small unilamellar liposomes with different compositions. We found β_2GPI abundantly binding to negatively charged liposomes containing 30% phosphatidylserine (PS) or 30% phosphatidylglycerol (PG). Considering the strong association of β_2GPI with liposomes containing 30% PS or 30% PG and the fact that these liposomes are cleared much more rapidly than neutral ones, we studied the possible involvement of β_2GPI in the interaction of liposomes with HepG2 cells, which are human hepatoma cells resembling hepatocytes, as well as with rat hepatocytes, Kupffer cells and liver endothelial cells.

2. Materials and methods

2.1. Chemicals

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (PS) and L-α-phosphatidylglycerol (egg, chicken-sodium salt) (PG) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (CH) and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). [1α,2α(*n*)-^{3}H]cholesteryl oleyl ether ([^{3}H]-COE) was obtained from Amersham (Buckinghamshire, UK). 1,1′ Dioctadecyl-3,3,3′ ,3′ -tetramethyl indocarbocyanine perchlorate (DiI) was purchased from Molecular Probes (Eugene, OR). Bio-Gel A-1.5 m gel was from Bio-Rad (Hercules, CA). Human β_2GPI was purified according to Ref. [15], and was a generous gift from Dr. E.M. Bevers (Cardiovascular Research Institute Maastricht, Maastricht University). Collagenase, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) were from Roche Diagnostics (Mannheim, Germany). All other chemicals were analytical grade or the best grade available.

2.2. Animals

Specified pathogen-free (SPF) male Wag/Rij rats (Harlan, Horst, The Netherlands) were used for isolation of hepatocytes, Kupffer cells and liver endothelial cells. The animals received humane care and experimental protocols were approved by the local committee for care and use of laboratory animals.

2.3. Methods

2.3.1. Preparation of liposomes

Lipids from stock solutions of PC, PS, CH and PG in chloroform/methanol (9:1) were mixed in the following molar ratios: PC/CH (60:40), PC/CH/PS (50:40:10), PC/CH /PS (45:40:15), PC/CH/PS (40:40:20), PC/CH/PS (35:40: 25), PC/CH/PS (30:40:30), PC/CH/PG (50:40:10) and PC/ CH/PG (30:40:30). The lipid mixtures were dried under reduced nitrogen pressure, dissolved in cyclohexane and lyophilized. The lipids were then hydrated in HN buffer [10 mM *N*-2-hydroxyethylpiperazine-*N*′ -2-ethanesulfonic acid (HEPES), 135 mM NaCl, pH 7.4] and vortexed. The liposomes formed were sized by repeated extrusion (13 times) through polycarbonate filters (Costar, Cambridge, MA), pore size 50 nm, using a high-pressure extruder (Lipex, Vancouver, Canada). When appropriate, 0.5 or 1 mol% of DiI was added to the lipid mixtures as a fluorescent marker. When required, liposomes were radiolabeled with a trace amount of [^{3}H]-COE, which was included in lipid mixture during liposome preparation. Phospholipid phosphorus of each liposome preparation was determined by phosphate assay after perchloric acid destruction [16]. Liposome size and size distribution were analyzed by dynamic light scattering, and zeta potential was determined by electrophoretic light scattering using a Nicomp Model 380 Submicron Particle analyzer (NICOMP particle sizing systems, Santa Barbara, CA). The diameter of the liposome preparations used, which was obtained from the volume distribution curves produced by the particle analyzer, was around 80 nm. No differences in liposome diameter were observed between liposomes with different compositions.

2.3.2. Separation of liposomes from serum using spin columns

Liposomes (5 mM liposomal lipid) were incubated with 70% human or rat serum at 37 °C for 1 h. The liposomes were then isolated from the serum by a spin column procedure [17] with minor modifications. Briefly, 1-ml tuberculin syringes plugged with glass wool were filled with Bio-Gel A-1.5 m gel equilibrated with HN buffer (pH 7.4) and centrifuged. Several fills and centrifugations were performed until the bed volume approximated 1 ml. The aliquots of the incubation mixture (50 μl) were then applied to spin columns and immediately centrifuged (800 rpm, 1 min). Column fractions were collected in glass tubes by applying 50 μl of HN buffer to the columns and subsequent centrifugation (800 rpm, 1 min). The liposome content of the column fractions was determined by measuring DiI-fluorescence with a FL500 fluorescence plate reader (Bio-Tek® Instruments, Winooski, VT) or by measuring the radioactivity associated with the fractions. The amount of adsorbed serum proteins was measured by protein determination [18].

2.3.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein adsorption to liposomes

Equal amounts of liposomes (30 nmol total liposomal lipid) isolated from serum incubations (see above) were applied to a 10% SDS-polyacrylamide gel. The proteins associated with liposomes were separated by SDS-PAGE using the Mini Protean-III apparatus (Bio-Rad) under reducing conditions. Proteins were then visualized by silver staining [19]. The silver-stained gels were scanned with Bio-Rad GS-710 Calibrated Imaging Densitometer and the

intensity of the protein bands was quantified using software Quantity One®.

2.3.4. Western blot analysis of liposome-associated β_2GPI

Equal amounts of liposomes isolated from serum incubations were applied to a 10% SDS-PAGE gel and the proteins associated were separated as described above. The proteins were then electro-transferred to nitrocellulose membranes (Bio-Rad) overnight at 4 °C using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad) at a constant current of 100 mA according to the manufacturer's instructions. The blots were blocked for 2 h with 2% BSA in TBST buffer (100 mM Tris–Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). Blots were subsequently incubated for 3 h at room temperature with rabbit anti-human β_2GPI (1:2000, Nordic) in TBST buffer containing 1% BSA. This was followed by washing and incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) and the detection of signals with NBT/BCIP developing solution.

2.3.5. Cell isolation and culture

Rat hepatocytes, Kupffer cells and liver endothelial cells were isolated after collagenase perfusion [20], followed by centrifugation and counterflow centrifugal elutriation as described previously [21]. After isolation, hepatocytes were cultured in 24-well plates in Williams E medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Kupffer cells were grown in 24-well plates in RPMI-1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Liver endothelial cells were cultured in collagen-coated 24-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) in RPMI-1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml endothelial cell growth factor (Boehringer, Germany). The freshly isolated liver cells were all maintained in a humidified 5% CO_2/95% air atmosphere at 37 °C. The media, now containing 10% FCS, were refreshed after 20 h and every 24 h thereafter. Experiments with isolated liver cells were performed on the third day after isolation of the cells.

In addition, human hepatoma HepG2 cells were grown in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2/95% air atmosphere at 37 °C.

2.3.6. Uptake and binding studies

Liposomes labeled with [^{3}H]-COE were first incubated with human serum or rat serum or human β_2GPI (400 μg/ml) for 1 h at 37 °C. Then the liposomes were separated from the free proteins with spin columns. Fractions 5–7 (Fig. 1) were used for uptake and binding studies. One hour before the start of the experiment, HepG2 cells, hepatocytes, Kupffer cells or liver endothelial cells were washed and preincubated with serum-free medium. Subsequently, the medium was removed and replaced by serum-free medium containing 160 nmol/ml untreated control liposomes or liposomes pretreated with serum or β_2GPI and incubated for 3 h at 37 °C. After the incubation, the culture plates were placed on ice and the medium was removed. The cells were rinsed six times with ice-cold phosphate buffered saline, pH 7.4. The cells were then lysed in 0.4 M NaOH and cell-associated radioactivity was determined by scintillation counting. Protein content was measured according to Lowry [22].

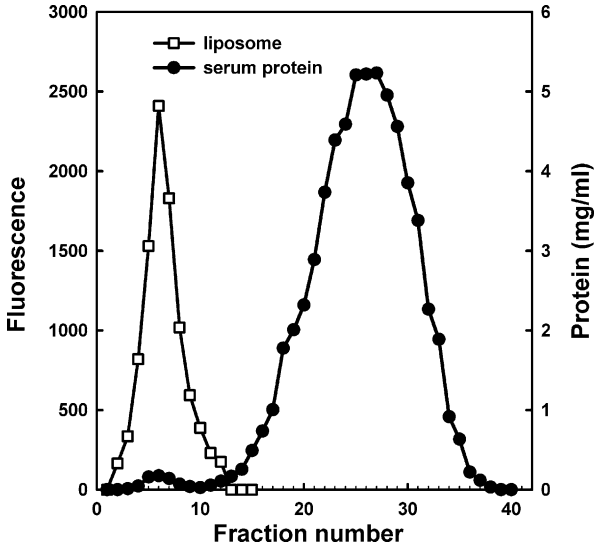

Fig. 1. Elution profile of liposome/serum incubation mixtures. Liposomes labeled with DiI were incubated with human serum at 37 °C for 1 h. Fifty-microliter aliquots of the incubation mixtures were then chromatographed on spin columns as described in Materials and methods. Open symbols represent DiI-fluorescence intensity (arbitrary units), and filled symbols represent the protein content of the column fractions.

2.3.7. Statistical analysis

Statistical significance of differences was evaluated by a two-tailed unpaired Student's *t*-test.

3. Results

3.1. Protein adsorption patterns of differently composed liposomes and identification of a ~55-kDa protein

To determine the profiles of protein adsorption to the liposomal surface, liposomes of different compositions were incubated with human serum at 37 °C and then separated from serum. As shown in Fig. 1, liposomes were effectively separated from serum proteins using the spin column method. Typically, liposomes were eluted at fractions 5 to 8 in which no proteins could be detected when serum alone was loaded onto the columns. Thus, in all our experiments, after isolating liposomes from incubation mixtures, fractions 5–7, which contained the highest amount of liposomes, were used for further analysis. Table 1 presents data on the

Table 1
Liposome characterization and the amount of proteins associated with differently composed liposomes during in vitro incubation with human serum (n=3, mean±S.D.)

Lipid composition	Size (nm)	Zeta potential (mV)	Protein association (μg protein/μmol total lipid)
PC/CH (60:40)	80.6±3.3	−1.18±1.89	23.9±3.4
PC/CH/PS (50:40:10)	78.4±1.8	−5.71±5.01	27.6±3.7
PC/CH/PS (30:40:30)	81.8±7.0	−39.81±6.85	85.2±3.4
PC/CH/PG (50:40:10)	74.5±4.7	0.32±2.19	28.2±4.6
PC/CH/PG (30:40:30)	76.4±3.6	−32.67±5.08	85.1±13.4

amounts of human serum proteins adsorbing to differently composed liposomes. The relative abundance of the amounts of adsorbed proteins is in the following order: 30% PS/PG>10% PS/PG>neutral liposome. Fig. 2A and B show the protein adsorption patterns of differently composed liposomes. As shown in both panels A (human serum) and B (rat serum), a protein (as indicated by arrows), with an Mr of approximately 55 kDa, is abundantly associated with liposomes containing 30% PS or 30% PG, but not with liposomes containing no PS/PG (neutral) or only 10% PS/PG.

It appears that the origin of the serum, i.e., human or rat, has no significant influence on the protein adsorption patterns

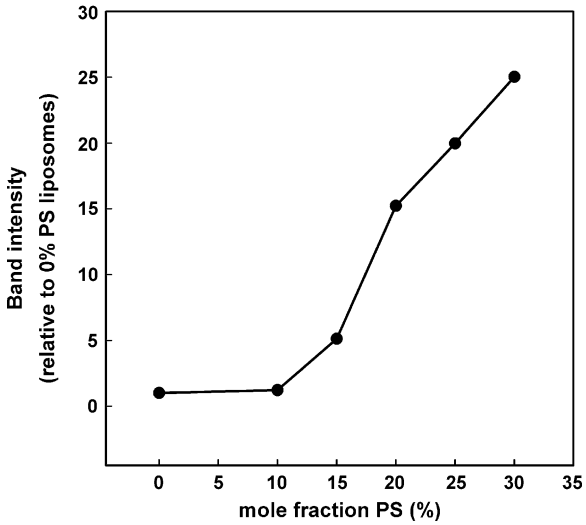

Fig. 3. Effect of PS concentration on 55-kDa protein adsorption to liposomes. The proteins associated with the recovered liposomes containing increasing amounts of PS (0–30 mol%) were separated on a 10% SDS-PAGE gel under reducing conditions and visualized by silver staining. Equal amounts of liposomal lipid were loaded onto each lane. The intensity of the 55-kDa protein bands was quantified using Quantity One® software.

of most liposome formulations used except for one protein. We repeatedly observed a protein (~50 kDa) associated in relatively high quantity with 30% PS-containing liposomes

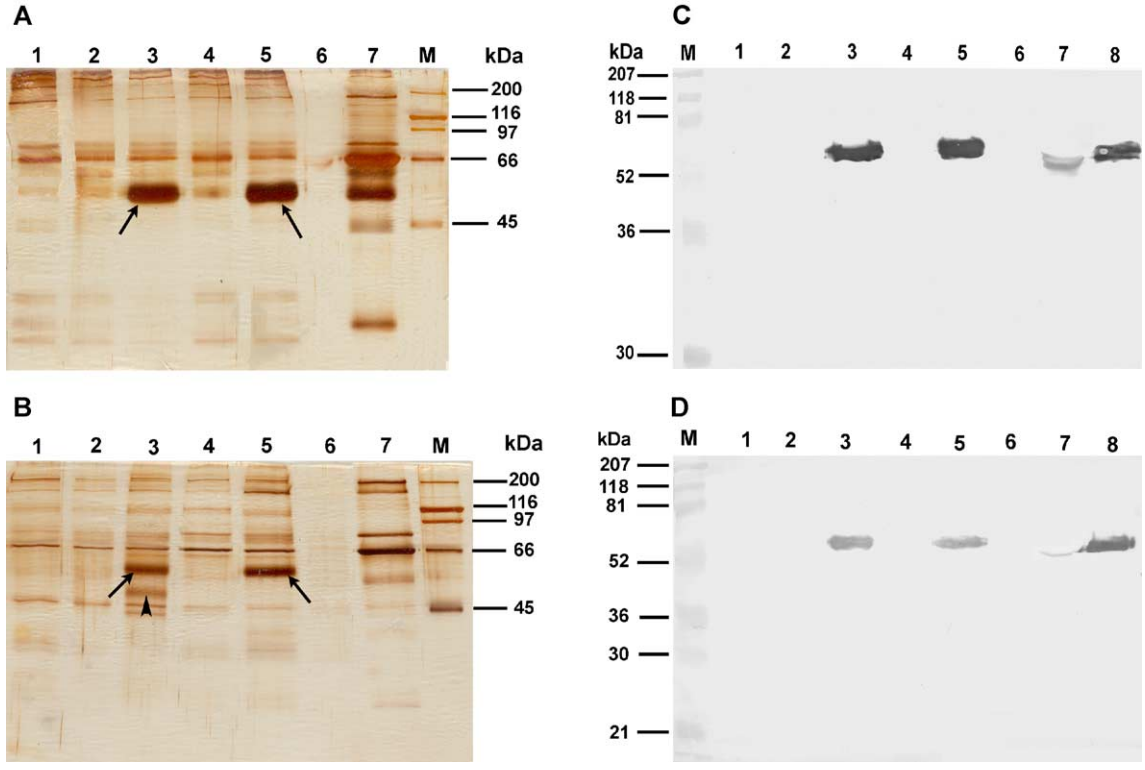

Fig. 2. Protein adsorption patterns (A, B) and Western blot analysis of adsorbed β_2GPI (C, D) of liposomes incubated with human serum (A, C) and rat serum (B, D). The proteins associated with the recovered liposomes (equal amounts of liposomal lipid) were separated on a 10% SDS-PAGE gel under reducing conditions, and were subsequently either visualized by silver staining (A, B) or immunoblotted for β_2GPI using rabbit antiserum against human β_2GPI (C,D). Lanes 1–5 represent proteins associated with PC/CH (60:40), PC/CH/PS (50:40:10), PC/CH/PS (30:40:30), PC/CH/PG (50:40:10), and PC/CH/PG (30:40:30) liposomes, respectively; lane 6, control (no liposomes, only serum was applied to spin columns and fractions 5–7 were collected and subjected to the SDS-PAGE gel); lane 7, human serum (A, C) or rat serum (B, D); lane 8, purified human β_2GPI; lane M, protein markers.

isolated from incubation with rat serum (as indicated by the arrow head in Fig. 2B), but we never observed this when liposomes were incubated with human serum. The identity of this protein has not yet been determined.

Since the ~55-kDa protein bound abundantly to liposomes with high negative charge density, and in view of an earlier observation reported by Chonn et al. [14], we argued that this protein might be β_2GPI. Western blot analysis using an anti-human β_2GPI antibody confirmed this (Fig. 2C). The abundantly binding protein originating from rat serum is likely to represent the rat homologue of human β_2GPI since it cross-reacted with the antibody against human β_2GPI (Fig. 2D).

The relation between amount of β_2GPI adsorbed and PS content of the liposomes is shown in Fig. 3. The amount of β_2GPI bound to liposomes gradually increases with PS concentration in the bilayer, as indicated by the results of the semi-quantitation of the 55-kDa bands by means of densitometry (Fig. 3). Clearly, the affinity of this protein for liposomes strongly increases with the concentration of the anionic phospholipid, once above 10 mol%.

3.2. Uptake and binding studies do not support a critical role of β_2GPI in liposome–hepatocyte interaction

To determine the role of β_2GPI in liposome–hepatocyte interaction, we performed uptake and binding experiments using HepG2 cells, a human hepatoma cell line, as well as isolated rat hepatocytes, Kupffer cells and hepatic endothelial cells. The liposome association with the cells after 3 h of incubation was compared with the cell association of untreated neutral liposomes, i.e., percentage of neutral control (Fig. 4). As indicated in Fig. 4A and B, pretreating liposomes with serum did not significantly influence the lipid association of the liposomes with HepG2 cells or primary cultures of rat hepatocytes, except in the case of liposomes containing 30% PS or 30% PG, but even the effect on these liposomes was inhibitory rather than enhancing. Pretreating liposomes with β_2GPI also inhibited the lipid association of liposomes containing 30% PS or 30% PG with HepG2 cells, but in the case of hepatocytes this inhibitory effect was only significant on the uptake of liposomes containing 30% PS not PG. In Kupffer cells and liver

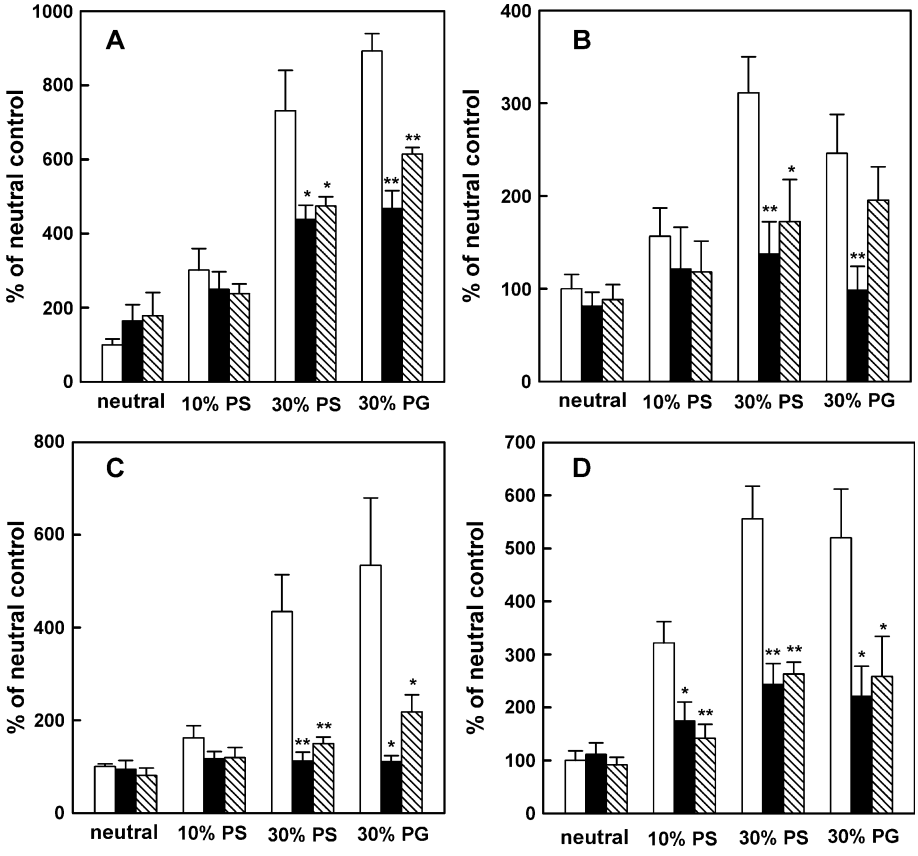

Fig. 4. Effect of human serum, rat serum or β_2GPI on the association of liposomes with HepG2 cells (A), hepatocytes (B), Kupffer cells (C) and liver endothelial cells (D). Liposomes were separated from human serum or rat serum or β_2GPI using spin columns after incubation at 37 °C for 1 h. The recovered or control liposomes at 160 nmol/ml were incubated with cells for 3 h, and uptake and binding were measured as described in Materials and methods. Control liposomes (open bars); liposomes pretreated with human serum (HepG2 cells) or rat serum (isolated rat liver cells) (filled bars); liposomes pretreated with β_2GPI (hatched bars). Data are expressed as percentage of neutral control (untreated neutral liposomes). Results are the mean±S.E. of three independent experiments each carried out in triplicate, except panel D which is from three independent experiments carried out in duplicate. 100% values for the association of untreated neutral liposomes with HepG2 cells, hepatocytes, Kupffer cells and liver endothelial cells are 0.74±0.11, 2.97±0.75, 3.45±0.36, 3.95±0.78 nmol of liposomal lipids/mg of cell protein, respectively. *P<0.05, **P<0.001, versus neutral control.

endothelial cells, preincubation of liposomes with serum or β_2GPI resulted in a significantly decreased association of liposomes containing 30% PS or 30% PG (Fig. 4C and D). Moreover, in liver endothelial cells, pretreatment of liposomes with serum or β_2GPI also led to a significant reduction in uptake and binding of 10% PS liposomes.

4. Discussion

Previous work in our laboratory has demonstrated the active involvement of hepatocytes in the elimination of liposomes from the blood [3,23]. Small liposomes (<100 nm) have relatively easy access to hepatocytes because they can pass through the fenestrae in the endothelial cell lining, thus extravasating from the hepatic microcirculation [3,24], although size is not the only parameter determining whether liposomes have access to hepatocytes [25]. Spanjer et al. [3] have shown that in rats small neutral liposomes (egg-PC/CH) are predominantly taken up by hepatocytes (95%), while of the hepatic uptake of the sphingomyelin/CH/PS formulation only 50% was accounted for by this cell type, indicating the importance of lipid composition in determining the extent to which the liposomes are taken up by hepatocytes. However, the mechanisms by which the liposome composition and size influence its in vivo uptake by hepatocytes are not completely clear. It has been proposed by us that serum proteins may play a role in making contact between liposomes and hepatocytes via certain receptor(s) on hepatocytes [4]. Upon exposure to blood, liposomes of different compositions attract different amounts and types of proteins, which may lead to different elimination kinetics and tissue distribution. In this study, we observed that the amount of protein adsorbing to the liposomal surface correlates with the density of negative charge of the liposomes (Table 1). Moreover, we clearly show here that the differently composed liposomes exhibit different serum protein adsorption patterns (Fig. 2A and B). A consistent observation is that there is hardly any difference between liposomes containing 10% PS and neutral liposomes in protein adsorption pattern. Nonetheless, in vivo liposomes containing 10% PS are cleared much more rapidly from the blood than neutral liposomes. The seemingly contradictory in vitro and in vivo results are likely due to the existence of multiple uptake mechanisms. In addition to serum proteins, phospholipid headgroups may be an important factor in determining the fate of liposomes. Macrophages and endothelial cells might directly recognize the PS headgroups, possibly via scavenger receptors, thus leading to vesicle endocytosis [26,27].

β_2GPI, also known as apolipoprotein H, has been found to act as a cofactor for binding of antiphospholipid antibodies to membranes containing cardiolipin or PS [28,29]. The physiological function of β_2GPI is not completely clear. Anti-phospholipid antibodies have high prevalence in patients with autoimmune disorders, and are strongly associated with thrombosis and recurrent fetal loss [30]. Chonn et al. [14] reported high affinity binding of β_2GPI to large unilamellar liposomes containing 20% PS or 20% phosphatidic acid or 10% cardiolipin. They further showed that the circulation time of cardiolipin-containing liposomes was extended in mice pretreated with anti-β_2GPI antibodies, suggesting an important role of β_2GPI in clearance of these liposomes. In addition, Balasubramanian et al. [31] and Balasubramanian and Schroit [32] showed that β_2GPI plays a role in macrophage recognition of PS-containing liposomes. Since β_2GPI binds to 30% PS/PG-containing small liposomes in a very large quantity and hepatocytes are involved in the uptake of PS-containing small liposomes [3], it was tempting to speculate that this protein might enhance the clearance of negatively charged small liposomes by hepatocytes. However, our uptake and binding studies with HepG2 cells or hepatocytes do not support such a role for this protein. Similarly, we found no enhancing effect of β_2GPI on uptake of 30% PS/PG liposomes by Kupffer cells or liver endothelial cells either (Fig. 4). As a matter of fact, in all the cell association experiments we performed, we observed an inhibitory, rather than an enhancing, effect of β_2GPI on liposome uptake and binding of 30% PS/PG-containing liposomes, and the β_2GPI effect seems to correlate with serum effect. It has been demonstrated that a highly positively charged amino acid sequence, Lys282-Asn-Lys-Glu-Lys-Lys287, located in the fifth domain of β_2GPI, is critical for its binding to anionic phospholipids [33–35]. We have shown previously that negatively charged liposomes containing PS are likely to bind to cells by charge–charge interactions [26]. Therefore, the inhibitory effect of β_2GPI on the cellular uptake and binding of liposomes containing 30% PS/PG might be due to the shielding of the PS moiety by the adsorption of β_2GPI.

In conclusion, we have demonstrated that despite the abundant adsorption of β_2GPI to negatively charged liposomes, the enhancing effect that Chonn and coworkers assign to this protein in the elimination of negatively charged liposomes by the liver is not supported by our in vitro data, which rather indicate that β_2GP1 bound to liposomes is inhibiting, instead of enhancing, liposome uptake by all liver cell types investigated.

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References

[1] J.A.A.M. Kamps, H.W.M. Morselt, P.J. Swart, D.K.F. Meijer, G.L. Scherphof, Massive targeting of liposomes, surface-modified with anionized albumins, to hepatic endothelial cells, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 11681–11685.

[2] A. Murao, M. Nishikawa, C. Managit, J. Wong, S. Kawakami, F. Yamashita, M. Hashida, Targeting efficiency of galactosylated liposomes to hepatocytes in vivo: effect of lipid composition, Pharm. Res. 19 (2002) 1808–1814.
[3] H.H. Spanjer, M. van Galen, F.H. Roerdink, J. Regts, G.L. Scherphof, Intrahepatic distribution of small unilamellar liposomes as a function of liposomal lipid composition, Biochim. Biophys. Acta 863 (1986) 224–230.
[4] G.L. Scherphof, J.A.A.M. Kamps, The role of hepatocytes in the clearance of liposomes from the blood circulation, Prog. Lipid Res. 40 (2001) 149–166.
[5] F. Bonte, R.L. Juliano, Interactions of liposomes with serum proteins, Chem. Phys. Lipids 40 (1986) 359–372.
[6] A. Chonn, S.C. Semple, P.R. Cullis, Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes, J. Biol. Chem. 267 (1992) 18759–18765.
[7] D. Liu, F. Liu, Y.K. Song, Recognition and clearance of liposomes containing phosphatidylserine are mediated by serum opsonin, Biochim. Biophys. Acta 1235 (1995) 140–146.
[8] H.M. Patel, Serum opsonins and liposomes: their interaction and opsonophagocytosis, Crit. Rev. Ther. Drug Carr. Syst. 9 (1992) 39–90.
[9] D.V. Devine, K. Wong, K. Serrano, A. Chonn, P.R. Cullis, Liposome-complement interactions in rat serum: implications for liposome survival studies, Biochim. Biophys. Acta 1191 (1994) 43–51.
[10] H. Harashima, T.M. Huong, T. Ishida, Y. Manabe, H. Matsuo, H. Kiwada, Synergistic effect between size and cholesterol content in the enhanced hepatic uptake clearance of liposomes through complement activation in rats, Pharm. Res. 13 (1996) 1704–1709.
[11] M.J. Hsu, R.L. Juliano, Interactions of liposomes with the reticuloendothelial system: II. Nonspecific and receptor-mediated uptake of liposomes by mouse peritoneal macrophages, Biochim. Biophys. Acta 720 (1982) 411–419.
[12] J.D. Rossi, B.A. Wallace, Binding of fibronectin to phospholipid vesicles, J. Biol. Chem. 258 (1983) 3327–3331.
[13] M. Murai, Y. Aramaki, S. Tsuchiya, Identification of the serum factor required for liposome-primed activation of mouse peritoneal macrophages. Modified alpha 2-macroglobulin enhances Fc gamma receptor-mediated phagocytosis of opsonized sheep red blood cells, Immunology 86 (1995) 64–70.
[14] A. Chonn, S.C. Semple, P.R. Cullis, Beta 2 glycoprotein I is a major protein associated with very rapidly cleared liposomes in vivo, suggesting a significant role in the immune clearance of "non-self" particles, J. Biol. Chem. 270 (1995) 25845–25849.
[15] G.M. Willems, M.P. Janssen, M.M. Pelsers, P. Comfurius, M. Galli, R.F. Zwaal, E.M. Bevers, Role of divalency in the high-affinity binding of anticardiolipin antibody-beta 2-glycoprotein I complexes to lipid membranes, Biochemistry 35 (1996) 13833–13842.
[16] C.J.F. Böttcher, C.M. Van Gent, C. Pries, A rapid and sensitive sub-micro phosphorus determination, Anal. Chim. Acta 24 (1961) 203–204.
[17] A. Chonn, S.C. Semple, P.R. Cullis, Separation of large unilamellar liposomes from blood components by a spin column procedure: towards identifying plasma proteins which mediate liposome clearance in vivo, Biochim. Biophys. Acta 1070 (1991) 215–222.
[18] G.L. Peterson, A simplification of the protein assay method of Lowry et al. which is more generally applicable, Anal. Biochem. 83 (1977) 346–356.
[19] H. Blum, H. Beier, H.J. Gross, Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels, Electrophoresis 8 (1987) 93–99.
[20] E. Casteleijn, H.C. van Rooij, T.J. van Berkel, J.F. Koster, Mechanism of glucagon stimulation of fructose-1,6-bisphosphatase in rat hepatocytes. Involvement of a low-Mr activator, FEBS Lett. 201 (1986) 193–197.
[21] T. Daemen, A. Veninga, F.H. Roerdink, G.L. Scherphof, In vitro activation of rat liver macrophages to tumoricidal activity by free or liposome-encapsulated muramyl dipeptide, Cancer Res. 46 (1986) 4330–4335.
[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[23] F. Roerdink, J. Regts, B. Van Leeuwen, G.L. Scherphof, Intrahepatic uptake and processing of intravenously injected small unilamellar phospholipid vesicles in rats, Biochim. Biophys. Acta 770 (1984) 195–202.
[24] D.D. Chow, H.E. Essien, M.M. Padki, K.J. Hwang, Targeting small unilamellar liposomes to hepatic parenchymal cells by dose effect, J. Pharmacol. Exp. Ther. 248 (1989) 506–513.
[25] T. Daemen, M. Velinova, J. Regts, M. de Jager, R. Kalicharan, J. Donga, J.J. van der Want, G.L. Scherphof, Different intrahepatic distribution of phosphatidylglycerol and phosphatidylserine liposomes in the rat, Hepatology 26 (1997) 416–423.
[26] J.A.A.M. Kamps, H.W.M. Morselt, G.L. Scherphof, Uptake of liposomes containing phosphatidylserine by liver cells in vivo and by sinusoidal liver cells in primary culture: in vivo–in vitro differences, Biochem. Biophys. Res. Commun. 256 (1999) 57–62.
[27] S.M. Moghimi, A.C. Hunter, Recognition by macrophages and liver cells of opsonized phospholipid vesicles and phospholipid headgroups, Pharm. Res. 18 (2001) 1–8.
[28] M. Galli, P. Comfurius, C. Maassen, H.C. Hemker, M.H. de Baets, P.J. Breda-Vriesman, T. Barbui, R.F. Zwaal, E.M. Bevers, Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor, Lancet 335 (1990) 1544–1547.
[29] H.P. McNeil, R.J. Simpson, C.N. Chesterman, S.A. Krilis, Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H), Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 4120–4124.
[30] S.S. Shapiro, The lupus anticoagulant/antiphospholipid syndrome, Annu. Rev. Med. 47 (1996) 533–553.
[31] K. Balasubramanian, J. Chandra, A.J. Schroit, Immune clearance of phosphatidylserine-expressing cells by phagocytes. The role of beta2-glycoprotein I in macrophage recognition, J. Biol. Chem. 272 (1997) 31113–31117.
[32] K. Balasubramanian, A.J. Schroit, Characterization of phosphatidylserine-dependent beta2-glycoprotein I macrophage interactions. Implications for apoptotic cell clearance by phagocytes, J. Biol. Chem. 273 (1998) 29272–29277.
[33] P.N. Del, Y.H. Sheng, E. Raschi, D.A. Kandiah, A. Tincani, M.A. Khamashta, T. Atsumi, G.R. Hughes, K. Ichikawa, T. Koike, G. Balestrieri, S.A. Krilis, P.L. Meroni, Human beta 2-glycoprotein I binds to endothelial cells through a cluster of lysine residues that are critical for anionic phospholipid binding and offers epitopes for anti-beta 2-glycoprotein I antibodies, J. Immunol. 160 (1998) 5572–5578.
[34] J. Hunt, S. Krilis, The fifth domain of beta 2-glycoprotein I contains a phospholipid binding site (Cys281-Cys288) and a region recognized by anticardiolipin antibodies, J. Immunol. 152 (1994) 653–659.
[35] Y. Sheng, A. Sali, H. Herzog, J. Lahnstein, S.A. Krilis, Site-directed mutagenesis of recombinant human beta 2-glycoprotein I identifies a cluster of lysine residues that are critical for phospholipid binding and anti-cardiolipin antibody activity, J. Immunol. 157 (1996) 3744–3751.