LDL Induced Association of Anionic Liposomes with Cells and Delivery of Contents as Shown by the Increase in Potency of Liposome Dependent Drugs

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Purpose. To establish whether anionic liposomes interact with the low-density lipoprotein (LDL) receptor, to determine the role of lipoproteins in this interaction, and whether the association causes functional delivery of encapsulated drugs.

Methods. The cell lines used were CV1-P and CHO wild type, both of which express the LDL receptor, and CHOldlA7, which lacks the LDL receptor. Cellular association of encapsulated methotrexate and fluorescein, labeled phosphatidylethanolamine in the lipid bilayer, was measured. Potency of three liposome dependent drugs (N-phosphonacetyl-L-aspartic acid, fluoroorotic acid, and methotrexate-γ-aspartate) was also measured by growth inhibition.

Results. Association of liposomes containing at least 75 mol egg phosphatidylglycerol (ePG)/100 mol phospholipid with cells grown in defined medium supplemented with 1.0 mg/ml LDL was up to 30-fold higher with CV1-P or CHO wild type cells than with CHOldlA7, and 5-fold higher than association in defined medium lacking LDL. The addition of LDL did not yield any elevation of cellular association of distearoylphosphatidylglycerol liposomes. Increased association was paralleled by a corresponding increase in potency of all three liposome dependent drugs tested.

Conclusions. ePG liposomes interact with the LDL receptor in an LDL-dependent fashion, and the interaction results in the delivery of contents to cells.

KEY WORDS: liposome; drug delivery system; liposome stability; low-density lipoprotein.

INTRODUCTION

Liposomes are usually made from naturally occurring substances and are therefore regarded as being biocompatible and biodegradable. These favorable characteristics have led to the encapsulation of a wide variety of pharmaceutical agents for the purposes of targeted delivery to specific cells or to reduce toxicities associated with drugs exhibiting narrow therapeutic indices (1,2). The predominant route by which liposomes gain entry into cells is endocytosis via coated pits in a manner similar to that of a number of macromolecules with specific surface-bound receptors, such as low-density lipoprotein (LDL) (3). Because numerous cancer cells have increased LDL receptor activity compared to corresponding normal cells, it has become an attractive strategy to target delivery systems such as liposomes directly to the LDL receptor (4).

Upon IV injection, liposomes immediately come into contact with various plasma proteins which have a destabilizing effect upon lipid vesicles. Several investigators have shown that lipoproteins interact with liposomes and thereby cause leakage of their contents. The earliest of such interactions to be characterized was the destabilization of liposomes lacking cholesterol by high-density lipoprotein (HDL) (5). In contrast, we have demonstrated that even cholesterol containing liposomes, at concentrations relevant to drug delivery, leak their contents in the presence of serum. Leakage occurs for liposomes prepared from low-phase transition temperature anionic lipids, such as egg phosphatidylglycerol (ePG), but not for high phase transition temperature lipids, such as distearoylphosphatidylglycerol (DSPG) and dipalmitoylphosphatidylglycerol (DPPG), or for neutral phospholipids. Additionally, serum caused liposome leakage only when 75–100 mol% of the phospholipid was anionic. When specific lipoproteins were studied at their normal serum concentrations, leakage from ePG liposomes was greatest with LDL suggesting that LDL is the major cause of serum-induced leakage (6).

The fortuitous behavior of ePG liposomes to exhibit an interaction with LDL raises the possibility that this ePG liposome-LDL interaction can be utilized to deliver a pharmaceutical cargo to cells via the LDL receptor. Lundberg *et al.* have covalently attached apoB to liposomes for this purpose, but the spontaneous interaction with anionic vesicles discussed here is a simpler means to achieve this end (7). Therefore, in the present study we have chosen to investigate the association and potency of various anionic liposomal formulations in a chemically defined, lipoprotein, and apolipoprotein free growth medium in an attempt to understand the role of the LDL receptor in targeted drug delivery mediated by anionic liposomes.

MATERIALS AND METHODS

Reagents

ePG, DPPG, DSPG, and egg phosphatidylcholine (ePC) were obtained from Avanti Polar Lipid (Birmingham, AL) and stored as a chloroform solution at –20°C under argon gas in sealed ampules. Cholesterol, methotrexate (Mtx), fluoroorotic acid (FO), folic acid, reduced nicotinamide adenine dinucleotide phosphate (NADPH), and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO) (8). Dihydrofolic acid (DHF) was prepared from folic acid by the method of Blakley (9). Dihydrofolate reductase (DHFR) was isolated from the L1210 leukemia cell line by the method of Sirotnak *et al.* (10). LDL and HDL were isolated from fresh bovine blood, obtained from the Meat Science Laboratories (UW-Madison), based on the method of Burstein *et al.* (11).

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ABBREVIATIONS: apoB100, apolipoprotein B-100; apoB48, apolipoprotein B-48; CHOwt, CHO wild type; DPPG, dipalmitoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; ePC, egg phosphatidylcholine; ePG, egg phosphatidylglycerol; FO, fluoroorotic acid; HDL, high density lipoprotein; LDL, low-density lipoprotein; Mtx, methotrexate; Mtx-γ-asp, methotrexate-γ-aspartate; PALA, N-phosphonacetyl-L-aspartic acid.

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Isolated lipoproteins were dialyzed for at least 24 h against Tris HCl to guard against polyanion contamination. Isolated lipoproteins were also characterized by their apolipoprotein content using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Protein content of the isolated lipoproteins was determined using a Bradford reagent (Bio-Rad, Melville, NY) (12). Defined medium was supplemented with lipoprotein at concentrations relevant to those normally found in serum. N-phosphonacetyl-L-aspartic acid (PALA) was provided by Dr. V. L. Narayanan of the Division of Cancer Treatment, National Cancer Institute (13). Methotrexate-γ-aspartate (Mtx-γ-asp) was a generous gift from Dr. J. R. Piper, Southern Research Institute (14). All other reagents for cell culture were obtained from Sigma and all solutions for cell culture were prepared in endotoxin free water.

Cell Culture

CV1-P, an African green monkey kidney cell line, was obtained from Dr. P. Berg, Stanford University (15). CV1-P cells were grown in defined medium consisting of Dulbecco's modified Eagles medium (Cellgro) containing 1 g/liter glucose, supplemented with 100 units/ml penicillin/streptomycin, 0.456 g/l glutamine, 0.288 g/l pyruvic acid, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 1 mg/ml salt fractionated bovine serum albumin, 7.3 μg/l biotin, 0.68 mg/l vitamin B12, and 88 μg/l linoleic acid.

CHOwt and CHOldlA7, a mutant which specifically lacks the LDL receptor, were obtained from Dr. M. Krieger, Massachusetts Institute of Technology (16). Both CHO cell lines were grown in defined medium consisting of RPMI 1640 (Cellgro), supplemented with 100 units/ml penicillin/streptomycin, 0.456 g/l glutamine, 0.288 g/l pyruvic acid, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 1 mg/ml salt fractionated bovine serum albumin, 7.3 μg/l biotin, 0.68 ng/l vitamin B12, 8.9 mg/l l-alanine, 14.5 mg/l proline, 0.27 mg/l thioctic acid, 0.161 mg/l putrescine hydrochloride and 88 μg/l linoleic acid.

Liposome Preparation

Large unilamellar vesicles were prepared under sterile conditions using the reverse phase evaporation (REV) technique (17). The phospholipid:cholesterol ratio for all liposomes was 2:1, and all preparations will be referred to subsequently by the phospholipid content followed by the drug encapsulated. For ePG, the solvent was diethyl ether and sonication and ether evaporation were performed at 36°C. For DPPG and DSPG, the solvent was isopropyl ether and sonication and evaporation were performed at 55°C. The lipid content of liposomes was determined by the method of Bartlett (18). Particle size for these vesicles was typically 0.1–1.0 μm, as seen by SEM (unpublished data).

Mtx and Mtx-γ-asp solutions were prepared for encapsulation in buffer containing 50 mM morpholinoethanesulfonic acid (MES), 50 mM N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 1 mM ethylenediaminetetraacetate (EDTA), adjusted to pH 7.2 with NaOH and adjusted to 290 mmol/kg with NaCl. The tonicity of all drug solutions was measured with an osmometer (Wescor, Logan, UT). Unencapsulated drug and residual ether were removed by gel chromatography on a 1 × 10 cm sterile Sephadex G-50 column eluted with buffer of identical composition. After solubilizing a small portion of the liposomes in 1:3:1 chloroform:methanol:water a spectrophotometric determination of drug concentration was made using an extinction coefficient of 7943 M^{-1} cm^{-1} at 370 nm.

FO solution was prepared at a concentration of 50 mM in buffer containing 50 mM MES, 50 mM morpholinopropanesulfonic acid (MOPS), 2 mM EDTA, adjusted to pH 7.4 with LiOH and adjusted to 290 mmol/kg with LiCl. Unencapsulated FO was removed by passage through a 1 × 10 cm sterile Sephadex G-75 column eluted with buffer of identical composition. After solubilizing a small portion of the liposomes in 1:3:1 chloroform:methanol:water a spectrophotometric determination of FO concentration was made using an extinction coefficient of 7100 M^{-1} cm^{-1} at 284 nm.

PALA solution was prepared at a concentration of 60 mM in buffer containing 50 mM MES, 50 mM HEPES, 1 mM EDTA, adjusted to pH 6.7 with NaOH and adjusted to 290 mmol/kg with NaCl. Calcein was also included in the PALA solution at a concentration of 2 mM and was used as an indicator of PALA encapsulation. Liposomes were separated from unencapsulated PALA by passage through a 1 × 10 cm sterile Sephadex G-75 column eluted with buffer of identical composition. After solubilizing a small portion of liposomes in 0.1% Triton X-100 the concentration of PALA was indirectly determined from the concentration of coencapsulated calcein. Spectrophotometric determination of calcein concentration was made using an extinction coefficient of 55053 M^{-1} cm^{-1} at 493 nm.

Liposome Aqueous Content Association

Liposome association studies were performed based on the method previously developed by Ng and Heath (19). Cells were plated in 24 well-culture plates (Corning, NY) in incubation medium at a concentration of 3×10^4 cells per ml, 1 ml per well, followed by overnight incubation at 37°C prior to the experiment. Triplicate wells were then treated for the indicated time with Mtx in either ePG or DSPG liposomes at a final Mtx concentration of 20 nM and final lipid concentration of approximately 200 nM. For samples tested in the defined medium supplemented with lipoprotein, liposomes were added to wells after a 30 min preincubation with lipoprotein. After incubation was complete, growth medium was gently aspirated, and the cells were washed twice with 0.5 ml of phosphate buffered saline containing 0.36 mM $CaCl_2$ and 0.42 mM $MgCl_2$ (PBS C/M) to remove unbound liposomes and Mtx. The cells were resuspended by treatment with 0.3 ml of 0.1% trypsin and 1 mM EDTA in phosphate buffered saline (PBS) at 37°C for 20 min, harvested, and transferred to a 1.5 ml eppendorf microtube. The tubes were placed in a boiling water bath for 15 min to release the cell associated Mtx (20).

The concentration of cell associated Mtx is determined by an enzyme inhibition assay involving the decrease in activity of L1210 DHFR in the presence of Mtx. DHFR activity is determined from the dihydrofolate dependent oxidation of NADPH at 340 nm. All reagents were dissolved in 0.1 M PBS, pH 7.4, 290 mmol/kg, 10% serum, and 10 mM DTT. In all cases, 0.2 ml samples were analyzed in triplicate at the same time as a series of standards containing 0 to 0.3 pmole of Mtx. Incubation times were controlled by adding reagents to successive tubes in 4 s intervals. Freshly prepared DHFR (0.25 mU) and NADPH (60 nmol) in 0.1 ml of buffer was added

followed by vortexing. Freshly prepared DHF (20 nmol in 0.1 ml of buffer) was added, the mixture was vortexed and placed in a 37°C water bath for 12 min. The reaction was stopped by adding 0.1 ml of 200 nM Mtx in buffer without serum. The absorbance of the samples was measured at 340 nm in a Beckman DU-64 spectrophotometer. The concentration of Mtx was determined by a plot of absorbance at 340 nm versus Mtx concentration; the plot was linear up to 0.3 nM Mtx and permitted measurement of 0.05–0.5 pmol Mtx. For cell association studies, the percent of added Mtx that associated with cells was calculated.

Liposome Lipid Association

Cells were plated in 24 well culture plates in incubation medium at a concentration of 3×10^4 cells per ml, 1 ml per well, followed by overnight incubation at 37°C prior to the experiment. Triplicate wells were then treated for one hour with fluorescein labeled liposomes incorporating 10 mol% L-α-phosphatidylethanolamine-N-fluorescein (PE-fluorescein, Avanti) in the lipid mixture. A final lipid concentration of 200 nM was utilized, which corresponds to the final lipid concentration in the liposome association studies. For samples tested in the defined medium supplemented with lipoprotein, liposomes were added to wells after a 30 min preincubation with lipoprotein. The cells were resuspended by treatment with 1.0 ml of 0.1% trypsin and 1 mM EDTA in PBS at 37°C for 20 min, harvested, and lysed with 100 μl of 10% (w/v) deoxycholate. Fluorescein fluorescence was determined (excitation/emission maxima 497/521 nm) using a Hitachi F-3010 fluorescence spectrophotometer. The concentration of lipid was determined by a plot of fluorescence intensity versus lipid concentration. Percent lipid associated is expressed as the percent of lipid originally added to the cell culture.

Growth Inhibition Studies

Cells were plated at 3×10^4 cells per ml, 1 ml per well, in 24 well plates. Following overnight incubation at 37°C, the cells were treated in triplicate with 10 μl of drug from a half-logarithmic dilution series. Three wells were used to obtain the original cell count and three wells were treated with 10 μl of buffer. The cells were allowed to grow for 72 h, the medium was aspirated, the cells were trypsinized, harvested, and counted in a Coulter model ZM counter. The concentration of drug that inhibits the cell count by 50% (IC_{50}) was determined from a plot of percent growth versus drug concentration.

RESULTS

The results of liposome-cell association studies utilizing (100 mol%) ePG encapsulated Mtx are summarized in Table I. The amount of association observed for incubation times between 10 and 60 min was quite constant with no specific trend during the 60 min period studied. Consequently, Table I shows the average amount associated for incubation periods up to 60 min. The addition of 0.1 mg/ml and 1.0 mg/ml LDL yielded approximately a 4- and 5-fold increase, respectively, in Mtx association in the CV1-P cell line. Similarly, the addition of 0.1 mg/ml and 1.0 mg/ml LDL yielded approximately a 6- and 7-fold increase, respectively, in association of Mtx with the CHOwt cell line. Conversely, the addition of LDL at either concentration had a negligible effect upon Mtx association with the CHOldlA7 cell line. The addition of HDL at either concentration did not have any substantial impact upon cellular association of Mtx with the CHOwt and CHOldlA7 cells. However, HDL caused nearly a two-fold elevation of ePG liposome association by CV1-P cells. The heightened ePG-Mtx association in the presence of LDL supports the theory that ePG liposomes utilize the LDL receptor and this route is directly responsible for the increased uptake seen in the wild type and CV1-P cell lines.

The results of liposome-cell association studies utilizing DSPG encapsulated Mtx are also summarized in Table I. Association was rapid and fairly constant over the incubation time with no specific trend during the 60 min period studied. Association of DSPG-Mtx to cell surface components is more extensive than the association observed with ePG-Mtx. However, the addition of LDL at either concentration tested did not enhance cellular association of DSPG-Mtx in any of the cell lines tested. Association was comparable in both CHO cell lines indicating that these liposomes do not utilize the LDL receptor for uptake. Addition of HDL did not significantly alter uptake in the CHOwt and CV1-P cells, but the presence of HDL at either concentration eliminated all detectable uptake in the mutant cell line. Taken as a whole the DSPG association studies imply that these liposomes do not extensively employ the LDL receptor for uptake, but uptake in the mutant cell line exists via an alternative pathway that is inhibited by HDL.

Liposome-cell association studies correlating the uptake

Table I. Cellular Association of ePG and DSPG Encapsulated Mtx[a]

		% Mtx associated ± standard deviation				
Lipid	Cell line	Control	LDL (0.1 mg/ml)	LDL (1.0 mg/ml)	HDL (0.1 mg/ml)	HDL (1.0 mg/ml)
ePG:	CV1-P	0.38 ± 0.08	1.35 ± 0.13[b]	2.01 ± 0.14[b]	0.59 ± 0.15	0.72 ± 0.13
	CHOwt	0.26 ± 0.20	1.43 ± 0.10[b]	1.84 ± 0.14[b]	0.26 ± 0.14	0.16 ± 0.16
	CHOldlA7	0.14 ± 0.19	0.12 ± 0.15	0.05 ± 0.08	0.04 ± 0.09	0.11 ± 0.11
DSPG:	CV1-P	0.61 ± 0.09	0.60 ± 0.08	0.67 ± 0.16	0.63 ± 0.18	0.63 ± 0.18
	CHOwt	0.46 ± 0.16	0.27 ± 0.14	0.32 ± 0.12	0.39 ± 0.15	0.46 ± 0.18
	CHOldlA7	0.37 ± 0.19	0.20 ± 0.15	0.20 ± 0.15	0	0

[a] The association of liposomal aqueous contents was determined in the presence of defined medium either alone (control), with 0.1 mg/ml LDL, with 1.0 mg/ml LDL, with 0.1 mg/ml HDL, or with 1.0 mg/ml HDL. Mtx was encapsulated in liposomes prepared from ePG or DSPG together with cholesterol at a phospholipid:cholesterol ratio 2:1. Cells were exposed to encapsulated Mtx at a final Mtx concentration of 20 nM, which corresponds to final lipid concentration of approximately 200 nM.

[b] $P < 0.01$ compared to control.

of liposomal aqueous contents as a function of anionic phospholipid content are shown in Fig. 1. In defined medium, for all cell lines tested, there was a low level of uptake for all formulations with at least 25 mol/100 mol ePG. Preparations with 0 mol/100 mol anionic phospholipid content displayed negligible amounts of Mtx uptake for all cell lines tested. The addition of HDL 1.0 mg/ml did not significantly alter the uptake of ePG-Mtx, regardless of anionic phospholipid content, for either of the CHO cell lines. In contrast, CV1-P displayed increasing levels of uptake, upon addition of 1.0 mg/ml HDL, as anionic content was increased. Similar results to those obtained with 1.0 mg/ml HDL were seen for all cell lines with the addition of 0.1 mg/ml HDL (data not shown). The enhanced uptake of 100 mol% ePG-Mtx upon addition of LDL 1.0 mg/ml seen in CHOwt and CV1-P also existed for formulations with at least 75 mol% ePG, but did not occur for liposomes with lower ePG content. Similar observations were made for these two-cell lines with LDL 0.1 mg/ml (data not shown). CHOldlA7 did not show an LDL mediated enhancement of uptake for any of the charge ratios tested.

As our previous studies monitored the fate of liposomal aqueous contents, equivalent lipid association studies were conducted to monitor the fate of liposomal lipid (Fig. 2). In defined medium, for all cell lines tested, there were low levels of uptake for all preparations. The addition of HDL 0.1 mg/ml (data not shown) or 1.0 mg/ml did not alter the lipid association properties in any of the cell lines. However, the addition of LDL 1.0 mg/ml resulted in a substantial increase in lipid association for 100 mol% (25-fold increase) and 75 mol% (23-fold increase) ePG vesicles in CV1-P. Comparable increases in lipid association were seen in CHOwt but not in CHOldlA7. Parallel studies utilizing LDL 0.1 mg/ml yielded similar findings (data not shown). Consistent with cell association studies (Table I), the addition of LDL at either concentration tested did not enhance lipid association of DSPG vesicles in any of the cell lines tested (data not shown). Liposomal lipid association for 75–100 mol% ePG in the presence of LDL is much higher than liposome content association. This is quite consistent with prior observations showing that LDL induces leakage of contents for these

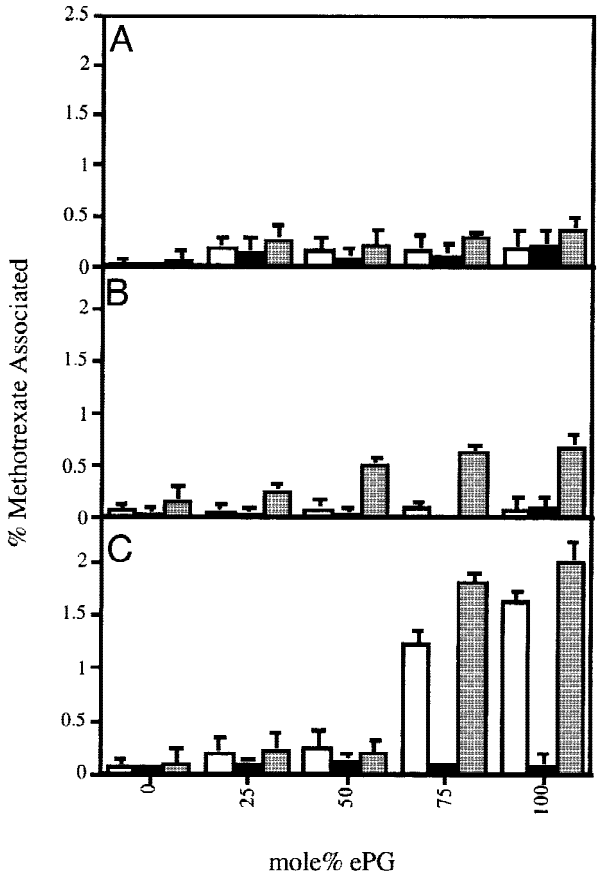

Fig. 1. Cellular association of liposome contents for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. The association of liposomal aqueous contents was determined in the presence of defined medium either alone (A), with 1.0 mg/ml HDL (B), or with 1.0 mg/ml LDL (C). Mtx was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid:cholesterol ratio 2:1. Cells were exposed for 60 min to encapsulated Mtx at a final Mtx concentration of 20 nM, which corresponds to a final lipid concentration of approximately 200 nM. Each bar is the mean of three determinations ± the standard deviation.

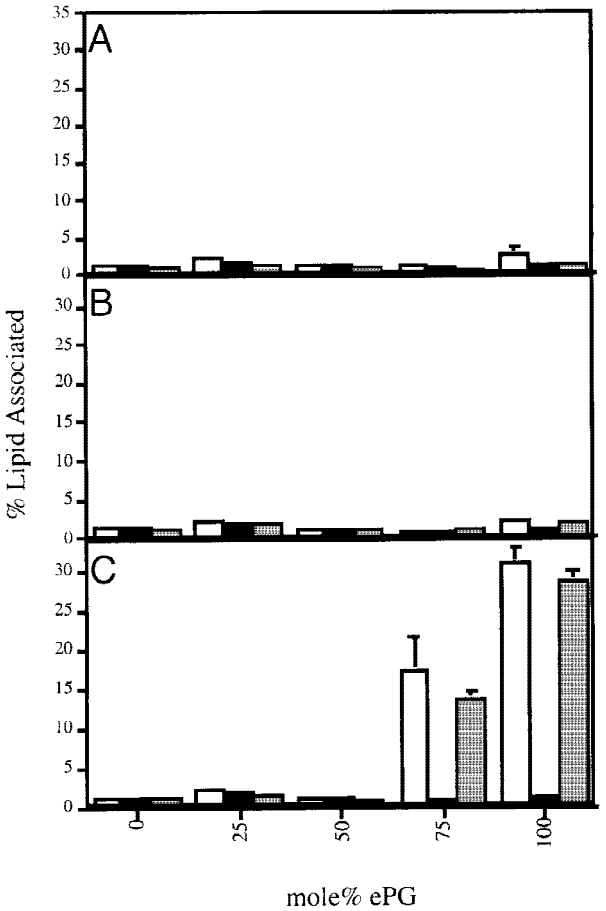

Fig. 2. Cellular association of liposomal lipid for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. The association of liposomal lipid was determined in the presence of defined medium either alone (A), with 1.0 mg/ml HDL (B), or with 1.0 mg/ml LDL (C). Fluorescein labeled liposomes were prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid:cholesterol ratio 2:1. Cells were exposed for 60 min to labeled vesicles at a final lipid concentration of 200 nM. Each bar is the mean of three determinations ± the standard deviation.

Table II. The Potency of ePG Encapsulated Liposome Dependent Drugs[a]

Cell line	Drug	IC_{50} (μM)				
		Control	LDL (0.1 mg/ml)	LDL (1.0 mg/ml)	HDL (0.1 mg/ml)	HDL (1.0 mg/ml)
CV1-P:	PALA	0.26 ± 0.03	0.04 ± 0.01[b]	0.05 ± 0.01[b]	0.21 ± 0.02	0.47 ± 0.13
	FO	0.86 ± 0.31	0.21 ± 0.03[b]	0.19 ± 0.02[b]	0.81 ± 0.04	0.71 ± 0.16
	Mtx-γ-asp	0.15 ± 0.04	0.06 ± 0.01[b]	0.04 ± 0.01[b]	0.15 ± 0.03	0.16 ± 0.02
CHOwt:	PALA	2.42 ± 0.22	0.88 ± 0.08[b]	0.38 ± 0.07[b]	2.55 ± 0.29	2.64 ± 0.28
	FO	4.82 ± 0.75	2.51 ± 0.64[b]	0.94 ± 0.45[b]	5.32 ± 0.61	5.30 ± 1.02
	Mtx-γ-asp	0.72 ± 0.11	0.30 ± 0.01[b]	0.13 ± 0.02[b]	0.71 ± 0.09	0.83 ± 0.06
CHOldlA7:	PALA	2.72 ± 0.23	5.50 ± 0.39	6.06 ± 0.10	3.37 ± 0.07	3.53 ± 0.14
	FO	6.00 ± 0.32	7.08 ± 0.32	6.41 ± 0.62	6.58 ± 0.57	5.44 ± 0.61
	Mtx-γ-asp	0.49 ± 0.06	0.52 ± 0.01	0.52 ± 0.01	0.55 ± 0.09	0.56 ± 0.02

[a] Growth inhibition was determined in defined medium either alone (control), with 0.1 mg/ml LDL, with 1.0 mg/ml LDL, with 0.1 mg/ml HDL, or with 1.0 mg/ml HDL. PALA/ePG lipid ratio = 0.440 mol/mol, FO/ePG lipid ratio = 0.437 mol/mol, and Mtx-γ-asp/ePG lipid ratio = 0.120 mol/mol.

[b] $P < 0.01$ compared to control.

two compositions. Based on the difference between contents and lipid association, we estimate the leakage to be 89% and 94% for 75 and 100 mol% ePG, respectively. These numbers closely agree with our prior measurements of leakage (6).

The growth inhibitory effects of ePG encapsulated PALA were determined in the presence of lipoproteins (Table II). The addition of LDL 0.1 mg/ml and 1.0 mg/ml yielded approximately a 7- and 5-fold increase, respectively, in potency in the CV1-P cell line. Similarly, the addition of LDL 0.1 mg/ml and 1.0 mg/ml enhanced potency approximately 3- and 6-fold, respectively, in the CHOwt cell line. The addition of LDL at either concentration led to approximately a two-fold decrease in potency on the CHOldlA7 cell line. The addition of HDL did not augment the potency of ePG-PALA for any of the cell lines tested. In the CHOldlA7 and CV1-P cell lines the addition of HDL actually led to a decrease in potency of the ePG-PALA formulation. Growth inhibition studies utilizing ePG encapsulated FO or Mtx-γ-asp produced similar findings, whereby LDL addition led to enhanced potency of ePG formulations on CHOwt and CV1-P but not on CHOldlA7, and HDL addition caused either minimal effect or a decrease in growth inhibition.

The growth inhibitory properties of DSPG encapsulated PALA were also determined in the presence of lipoproteins (Table III). The addition of LDL at either concentration did not result in the potency enhancement seen for the ePG formulations. In fact, the DSPG formulation exhibited a decrease in potency (CHOwt) or negligible effect (CV1-P) upon the addition of LDL to the growth medium. Similar experiments with DPPG encapsulated FO resulted in analogous findings. As with the ePG formulations, the DSPG preparation showed decreased potency upon lipoprotein addition in the CHOldlA7 cell line. Upon HDL addition at either concentration, DSPG encapsulated PALA exhibited no change in CHOwt or a decrease in potency as observed with CV1-P. Similar findings were observed in experiments with DPPG encapsulated FO.

Figure 3 displays results from growth inhibition studies comparing potency with the anionic phospholipid content of ePG vesicles. The LDL mediated enhancement in potency of the ePG-PALA preparation also occurred with vesicles containing 75 mol% ePG for CV1-P and CHOwt. There was no LDL enhanced potency for any of the formulations on CHOldlA7. The increased liposomal uptake seen in LDL supplemented defined medium for preparations containing at least 75 mol% ePG is consistent with the findings of these growth inhibition studies. Contrary to the LDL effect, addition of HDL did not yield an increase in potency for any of the charge ratios tested. Parallel growth inhibition studies with the liposome dependent drugs FO and Mtx-γ-asp gave analogous findings (Figs. 4 and 5).

DISCUSSION

The study described above documents one means by which anionic liposomes can interact with cells. Both liposome contents and lipid associate with CV1-P and CHOwt cells in a manner that appears to be dependent on the presence of LDL. Association occurs only for liposomes that contain 75–100 mol% ePG, and is not seen with CHOldlA7, a

Table III. Potency of DSPG Encapsulated PALA and DPPG Encapsulated FO[a]

Cell line	Drug	IC_{50} (μM)				
		Control	LDL (0.1 mg/ml)	LDL (1.0 mg/ml)	HDL (0.1 mg/ml)	HDL (1.0 mg/ml)
CV1-P:	PALA	0.15 ± 0.03	0.23 ± 0.03	0.23 ± 0.05	0.25 ± 0.01	0.19 ± 0.06
	FO	0.74 ± 0.20	0.73 ± 0.14	1.85 ± 0.18	2.01 ± 0.53	6.50 ± 0.30
CHOwt:	PALA	1.90 ± 0.34	3.77 ± 0.41	4.49 ± 0.94	1.93 ± 0.06	1.86 ± 0.25
	FO	6.28 ± 1.13	7.25 ± 0.71	6.92 ± 0.75	5.38 ± 1.03	5.49 ± 0.54
CHOldlA7:	PALA	2.31 ± 0.18	4.72 ± 0.27	5.72 ± 0.29	4.54 ± 0.52	6.26 ± 0.18
	FO	6.57 ± 0.54	7.23 ± 0.38	7.13 ± 0.25	6.87 ± 0.66	6.43 ± 0.54

[a] Growth inhibition was determined in defined medium either alone (control), with 0.1 mg/ml LDL, with 1.0 mg/ml LDL, with 0.1 mg/ml HDL, or with 1.0 mg/ml HDL. PALA/DSPG lipid ratio = 0.562 mol/mol and FO/DPPG lipid ratio = 0.451 mol/mol.

mutant cell line known to lack the LDL receptor. Drug delivery to CHOwt and CV1-P cells is also enhanced by the presence of LDL, but under only those circumstances where association is also increased. These results correlate closely with our earlier studies, which documented that the effects of serum on the leakage of drug from liposomes were caused primarily by an interaction with LDL. In those studies, leakage of liposome contents occurred only for liposomes prepared from low-phase transition temperature anionic lipids such as ePG, and the ePG content had to be 75–100 mol% to induce leakage (6). In the present study, enhancement of both association and drug delivery also occurs only for ePG liposomes containing more than 50 mol ePG/100 mol phospholipid.

From these observations, we may construct the following hypothesis for how LDL interacts with anionic liposomes and the consequent cell interaction:

Step 1. LDL binds to the liposomes via an electrostatic interaction that requires the presence of more than 50 mol ePG/100 mol phospholipid in the liposome bilayer. The binding of LDL to a highly anionic liposome may not involve the portion of LDL that binds to the receptor because polyanions such as heparin are known not to bind to the region of apoB100 responsible for binding to the LDL receptor (21). Similarly, it is plausible that LDL binds to these anionic vesicles in a manner such that the LDL receptor binding region remains accessible to the LDL receptor.

Step 2. The LDL particle coalesces with the liposome membrane, causing a substantial loss of liposome aqueous contents. This coalescence requires that the bilayer be in the liquid crystalline (fluid) state. The final product is a particle that retains an internal aqueous space and thereby retains some of the original liposome contents. Damen *et al.,* though working with neutral phospholipids, found that cholesterol containing liposomes were able to maintain their integrity in the presence of rat plasma or plasma fractions (22).

Step 3. The resultant hybrid particle binds to the cell membrane via an interaction of apoB100 with the LDL receptor. The delivery of the liposome-LDL complex to the LDL receptor seen in our system is analogous to the findings of Greenspan *et al.* in which they demonstrate that ePG vesicles can form a complex with LDL facilitating its phagocytosis by macrophages via scavenger receptors (23).

These observations are consistent with prior studies, both from this lab and from others. In their studies on the aggregation of DMPG liposomes by LDL, Lauraeus *et al.* suggested that an electrostatic interaction between the acidic phosphate of DMPG and cationic residues in apoB100 were responsible for the LDL induced aggregation of DMPG vesicles (24). Involvement of apoB100 was implicated by the finding that tryptic digestion of LDL abrogated its ability to cause aggregation. Interestingly, aggregation of DMPG liposomes by LDL was observed in the liquid crystalline state but not in the gel state. Additionally, increasing the content of DMPC in DMPG liposomes reduced aggregation and at 50 mol% anionic phospholipid content no aggregation was evident.

Despite the effect of LDL upon drug delivery, it should be mentioned that substantial drug delivery effects occur that are not induced by LDL. This effect is produced by a lower level of drug binding that occurs with liposomes prepared from DSPG and with ePG liposomes containing 25–50 mol ePG/100 mol phospholipid. The nature of this interaction or interactions remains to be characterized. However, the identification of the scavenger receptor SR-BI as an HDL receptor on the CHO cell lines provides one possible alternative pathway for the uptake seen in our studies (25). Additionally, gp330, a major kidney membrane protein and a member of

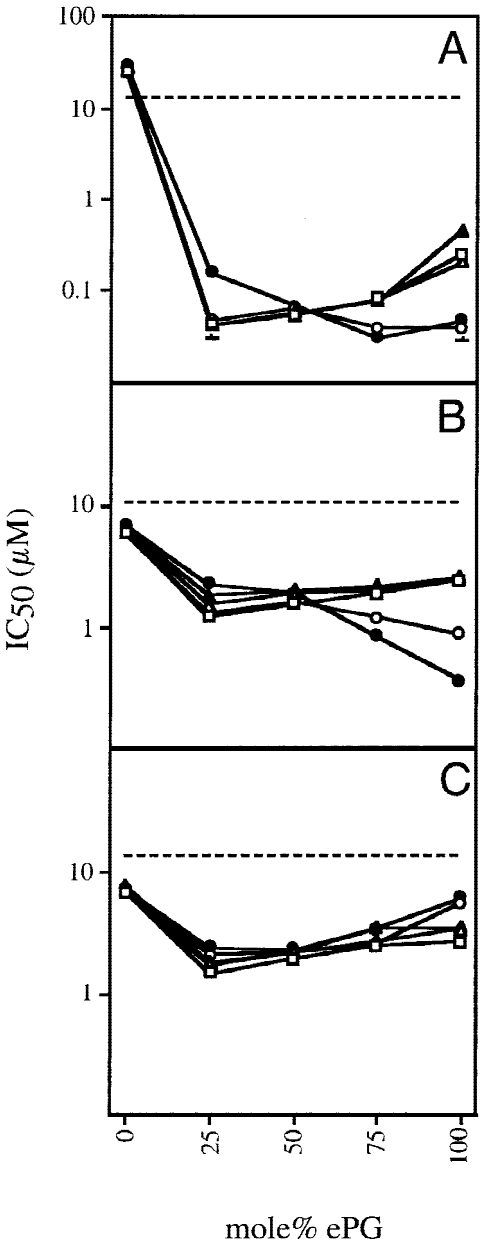

Fig. 3. The potency of liposome-encapsulated PALA for CV1-P (A), CHOwt (B), and CHOldlA7 (C) as a function of anionic phospholipid content. PALA was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid:cholesterol ratio 2:1. Potency was determined either in defined medium alone (□), with 0.1 mg/ml HDL (△), with 1.0 mg/ml HDL (▲), with 0.1 mg/ml LDL (○), or with 1.0 mg/ml LDL (●). The dotted line denotes the IC_{50} of free drug in the defined medium. Each point is the mean of three determinations ± the standard deviation. The drug lipid ratios were: 0 mol ePG/100 mol lipid, 0.434 mol/mol; 25 mol ePG/100 mol lipid, 0.735 mol/mol; 50 mol ePG/100 mol lipid, 0.452 mol/mol; 75 mol ePG/100 mol lipid, 0.412 mol/mol; 100 mol ePG/100 mol lipid, 0.440 mol/mol.

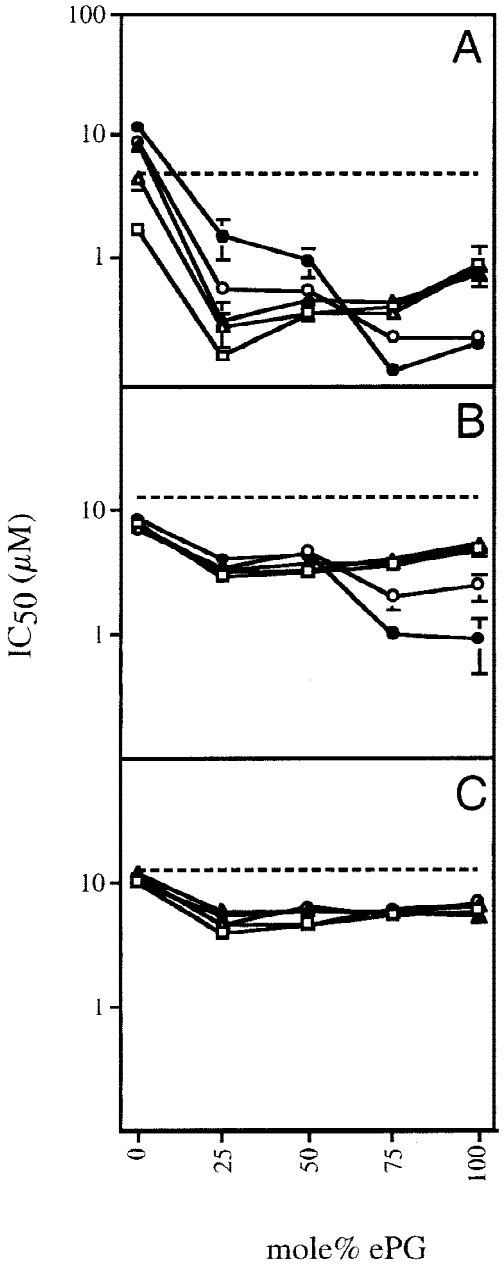

Fig. 4. The potency of liposome-encapsulated FO for CV1-P (A), CHOwt (B), and CHOldlA7 (C) as a function of anionic phospholipid content. FO was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid:cholesterol ratio 2:1. Potency was determined either in defined medium alone (□), with 0.1 mg/ml HDL (△), with 1.0 mg/ml HDL (▲), with 0.1 mg/ml LDL (○), or with 1.0 mg/ml LDL (●). The dotted line denotes the IC_{50} of free drug in the defined medium. Each point is the mean of three determinations ± the standard deviation. The drug lipid ratios were: 0 mol ePG/100 mol lipid, 0.607 mol/mol; 25 mol ePG/100 mol lipid, 0.673 mol/mol; 50 mol ePG/100 mol lipid, 0.547 mol/mol; 75 mol ePG/100 mol lipid, 0.689 mol/mol; 100 mol ePG/100 mol lipid, 0.437 mol/mol.

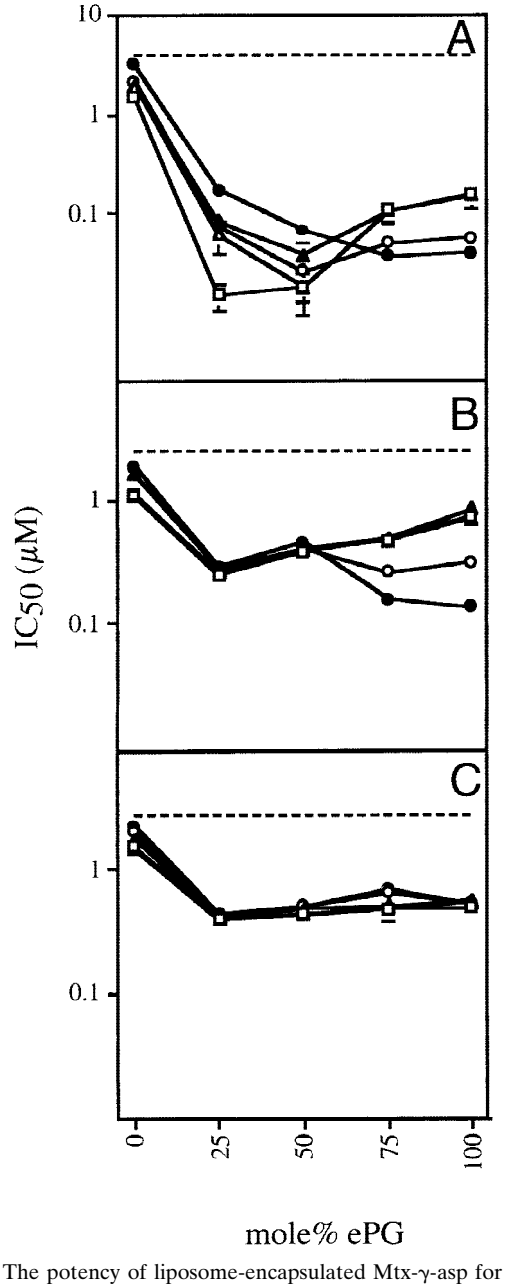

Fig. 5. The potency of liposome-encapsulated Mtx-γ-asp for CV1-P (A), CHOwt (B), and CHOldlA7 (C) as a function of anionic phospholipid content. Mtx-γ-asp was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid:cholesterol ratio 2:1. Potency was determined either in defined medium alone (□), with 0.1 mg/ml HDL (△), with 1.0 mg/ml HDL (▲), with 0.1 mg/ml LDL (○), or with 1.0 mg/ml LDL (●). The dotted line denotes the IC_{50} of free drug in the defined medium. Each point is the mean of three determinations ± the standard deviation. The drug lipid ratios were: 0 mol ePG/100 mol lipid, 0.241 mol/mol; 25 mol ePG/100 mol lipid, 0.151 mol/mol; 50 mol ePG/100 mol lipid, 0.145 mol/mol; 75 mol ePG/100 mol lipid, 0.132 mol/mol; 100 mol ePG/100 mol lipid, 0.120 mol/mol.

the LDL receptor gene family, may be involved in liposomal association in CV1-P cells (26). Other members of the LDL receptor gene family, such as LDL receptor related protein or cubulin, may be involved with liposome association but neither of these receptors has been identified on the cell lines used here.

It should be noted that for the LDL-mediated effect, the extent of lipid binding is much greater than the extent of drug binding. This is entirely in keeping with prior leakage studies, which showed that LDL induced an instantaneous loss of 70% of the contents of ePG liposomes (6). Hence, the leakage of contents would result in a lower fraction of liposome con-

tent association. As shown above, we have calculated leakage values based on the disparity between lipid and contents association and find values that are very similar to those previously measured (6). It should also be pointed out that DSPG liposomes leak very little in contact with serum or LDL. Consequently, the DSPG lipid and contents association will be similar and very low.

The nature of the effect of HDL on binding to CV1-P cells is not presently clear and does not lead to an increase in drug delivery. While the percentage of drug binding to CV1-P can be as much as half that induced by LDL, the level of lipid binding is much lower and is comparable to the extent of drug binding. This also is in keeping with the limited extent to which HDL induces leakage from these liposomes (6). The inhibition of association of liposomes with CHOldlA7 by HDL and LDL may well indicate that the form of association is mediated by the scavenger receptor SR-BI, discussed above.

The interaction of anionic liposomes with cells has been explored by a number of investigators (27,28). Unfortunately, it is difficult to compare our results to prior studies because our work has utilized a concentration of lipid relevant to our drug delivery studies, and this concentration is much lower than that used by other investigators. Consequently, it is quite possible that the LDL-mediated processes seen in our work might not have been a major part of association in other studies, particularly if the LDL receptors were to become saturated as lipid concentration is increased.

In conclusion, we have demonstrated that the interaction of LDL with fluid liposomes bearing at least 75 mol anionic phospholipid/100 mol phospholipid leads to cell association of the liposomes via an interaction with the LDL receptor. The association of the liposomes and their contents with cells subsequently produces drug delivery effects, manifested by an increase in the potency of encapsulated drug. We hope in future studies to explore the possible role of these interactions for *in vivo* drug delivery.

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