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Conjugation of apolipoprotein B with liposomes and targeting to cells in culture

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Mixed phospholipid/cholesterol (2:1 molar ratio) liposomes were conjugated with native and acetylated apolipoprotein B (apoB), the protein part of low density lipoprotein (LDL). The objective was to increase the specificity of the cellular uptake of liposomes by utilization of the LDL and scavenger receptor pathways. The method of choice for the conjugation of liposomes with apoB proved to be the detergent solubilization and removal procedure. Two detergents were tested;sodium cholate (NaC) and octyl glucoside (OG). The integrity of the resulting complexes was demonstrated by Sepharose CL-4B gel chromatography and Metrizamide gradient centrifugation. The conjugates showed a good physical stability and the leakiness was only marginally larger than for unconjugated liposomes. The interaction of apoB- and acetyl apoB–liposome conjugates with CV-1 and J774 cells, respectively, was monitored by an encapsulated pH-sensitive fluorophore, pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate (HPTS)). This dye provides means of detecting binding and endocytosis of conjugates in living cells. The internalization was a fast process and about 10-times faster for the OG-conjugates than for the corresponding unconjugated liposomes. The conjugates showed a clear concentration-dependent association of dye with cells, while this was less prominent with liposomes. The uptake was nearly an order of magnitude faster with CV-1 cells than with J774 cells. Acidification of intracellular conjugates proceeded fast during the first 30 min of incubation and reached a minimum value of approx. pH 6 after 3 h. The specificity of binding of apoB–liposome conjugates to CV-1 cells was demonstrated by displacement experiments with native LDL. The results indicate that apoB–liposome conjugates may be used as a delivery vehicle for bioactive subtances to cells.

Introduction

Liposomes have been extensively studied as a delivery system for biomedically active substances to cells in vitro and in vivo (for reviews, see Refs. 1,2). One of the central problems in using liposomes as drug carrier is the lack of target specificity. An attractive strategy to promote efficient and selective delivery of encapsulated molecules to cells is to utilize liposomes that are targeted to specific cell-surface receptors by means of liposome-conjugated proteins or other ligands [3].

In recent years, LDL has been considered as an endogenous drug carrier in cancer chemotherapy [4]. LDL is taken up by cells via a specific receptor that interacts with apoB on LDL [5]. The rationale for using LDL as a carrier for antineoplastic drugs is that many cancer cell lines have higher LDL receptor activity than the corresponding normal cells [6]. LDL can also be directed to non-lipoprotein receptors by chemical modification of apoB [7]. However, targeting by use of drug/LDL complexes can only be applied to lipophilic drugs, while a large majority of the compounds in use today are water-soluble. The present study offers a way to overcome this problem. The targeting properties of apoB are combined with the capacity of the liposomes to carry water-soluble compounds. Two major problems had to be overcome in this work. The method for conjugation should restore both the receptor binding properties of apoB and the integrity of the liposomes. This report describes a simple and reproducible method for the successful conjungation of native and modified apoB with liposomes and the biological activity of these complexes are tested on cells in culture. This system should have potential applications for effective delivery

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Abbreviations: apoB, apolipoprotein B; LDL, low density lipoprotein; NaC, sodium cholate; OG, octyl glucoside; HPTS, 8-hydroxy-1,3,6-pyrenetrisulfonate; POPC, palmitoyloleoylphosphatidylcholine; Na_2EDTA, etylenediaminetetraacetic acid disodium salt; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CF, carboxyfluorescein.

of bioactive substances to cells via receptor-mediated endocytosis.

Materials and Methods

Materials. Palmitoyloleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, AL, USA) and L-3-phosphatidylcholine,1-palmitoyl-2-[1-^{14}C]oleoyl (57 mCi/mmol) from the Radiochemical Centre (Amersham, UK). Cholesterol, n-octyl-β-D-glucopyranoside, sodium cholate and Metrizamide were obtained from Sigma (St. Louis, MO, USA). Pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid, disodium salt) was from Molecular Probes (Eugene, OR, USA), Bio-Beads SM-2 (20–50 mesh) from Bio-Rad Laboratories (Richmond, CA, USA) and carboxyfluorescein (CF) from Eastman Kodak (New York, NY, USA) and was purified on a Sephadex LH-20 column. The scintillation cocktail Liquiscint was obtained from National Diagnostics. All other chemicals were reagent grade.

Lipoprotein isolation. Plasma was obtained from freshly drawn blood from normal human volunteers. LDL (d = 1.019–1.063 g/ml) and high density lipoprotein (HDL) (d = 1.063–1.21 g/ml) was isolated by differential density ultracentrifugation using standard procedures [8]. Isolated LDL was washed by ultracentrifugal flotation through an overlayering solution of d = 1.063 g/ml. The centrifugations were performed in a Beckman SW-28 rotor at 27000 rpm at 4°C for 24 h. The purity of LDL was verified by agarose electrophoresis by staining with Oil Red O and Coomassie brilliant blue. The isolated LDL was dialyzed in the dark at 4°C for 24 h against 0.15 M NaCl and 0.05% Na_2EDTA (pH 7.4), filtered through a 0.22-μm filter and stored at 0°C in sterile ampoules. Acetylation of LDL was performed by reaction with acetic acid anhydride as described in Ref. 9. LDL was labelled with ^{125}I to a specific activity of about 100 cpm/ng using the IODO-GEN™ method [10] (Pierce, Rockford, IL, USA) and subsequently purified by Sephadex G-75 gel chromatography.

Delipidation of apoB. Prior to conjugation with liposomes, apoB, the protein component of LDL, was delipidated by extraction with heptane to remove endogenous neutral lipids, principally as described in Ref. 11. Briefly, 2.5 mg LDL in 400 μl was dialyzed at 4°C for 60 h against three changes of 3 liters of 0.3 mM Na_2EDTA (pH 7.0). Then sucrose was added to a final concentration of 25% (w/v) to stabilize the LDL-protein during lyophilization and heptane extraction. The solution was rapidly frozen and lyophilized over night. The residue was then stored under vacuum at 0°C in a desiccator containing P_2O_5 until it was completely dry (approx. 2 h). The dried LDL was extracted three times with 3 ml heptane at 0°C. The remaining heptane was evaporated in vacuum under argon at 0°C. The resulting delipidated apoB, which is freed of neutral lipids [12], was used for conjugation with liposomes as fast as possible.

Peparation and characterization of apoB–liposome conjugates. Liposomes were produced and conjugated with apoB by the detergent solubilization and removal method. The lipid components of the liposomes POPC and cholesterol (molar ratio 2:1) were mixed from stock solutions with 15 and 2 molar excesses (calculated on POPC basis) of OG and NaC, respectively, and evaporated to dryness under argon. After addition of buffer (2.5 mM Hepes, 75 mM NaCl, 50 μM EDTA (pH 7.4), standard buffer) the mixtures were subjected to a short sonication in a bath sonicator to ensure complete solubilization of the lipids. The mixed micelles were then added to the delipidated apoB in the proportion 2:1 (weight ratio) between POPC and apoB and the mixture was rotated gently until the solution was completely clear. The detergents were then removed from the mixtures by passage through a Bio-Beads SM-2 (20–50 mesh) column (Bio-Rad). This method has been shown to bring about a practically complete removal of detergents from a codispersion with phospholipids and protein [13]. The beads were wetted with the standard buffer before use and the moist beads were then weighed into disposable micro-columns in an amount enough to absorb the whole sample. The mixed micelle/apoB mixture was then added to the column and incubated at 0°C for 30 min. The product was eluted into a collecting tube by centrifugation of the micro-column for 2 min in a table-top centrifuge at approx. 400 × g. These procedures for extracting of detergents by Bio-Beads SM-2 were repeated once. At this stage the solutions were opaque indicating the formation of liposomes. The apoB–liposome conjugates were further purified by passage through a Sephadex G-75 column (1 × 15 cm). Since the size of aggregated apoB might equal that of small liposomes, the conjugates were also isolated by flotation in a Metrizamide gradient to assure their purity. The apoB–liposome complexes were applied in 20% Metrizamide and overlayered with 10% Metrizamide and standard buffer. The conjugates were recovered in the boundary between buffer and 10% Metrizamide. The size of the isolated complexes was measured by quasielastic laser light scattering on a Malvern system 4700 sub-micron particle analyzer (Malvern Instruments, Malvern, UK). The density of the conjugates was determined by density-gradient centrifugation in a linear 0–40% sucrose gradient. The leakage was assessed by dialysis of apoB–liposome conjugates with entrapped CF against a 100-fold excess of standard buffer for 24 h and measuring CF in the dialysate and CF remaining in conjugates after release with Triton X-100.

Cell culture. The macrophage-like cell lines J774 and CV-1, an established line of African green monkey kidney cells, were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose and 10% foetal bovine serum and incubated under 7% humidified CO_2 at 37°C. Cells were plated at a concentration of 10^6 cells per 9.6 cm^2 plastic culture dish 24 h prior to use.

Cell incubation with HPTS-containing apoB–liposome conjugates. HPTS-containing conjugates were prepared by using solutions of HPTS (35 mM) in standard buffer for preparation of conjugates. Unencapsulated HPTS was removed by the chromatography on a Sephadex G-75 column (1 × 15 cm) equilibrated with 150 mM NaCl and 5 mM Hepes (pH 7.4). Before incubation with HPTS-containing conjugates the growth medium was removed and the cells were washed twice with 2 ml 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 (PBS (pH 7.4)) supplemented with 0.4 mM calcium, 0.4 mM magnesium and 5 mM glucose (PBS-CMG). Before the start of incubation the HPTS-containing conjugates were diluted to 80 μM phospholipid in PBS-CMG and 0.5 ml was added to each culture dish (approx. $2 \cdot 10^6$ cells). After incubation in a humidified incubator at 37°C, cells were analyzed for fluorescence.

Fluorometry. Fluorescense was measured with a SPEX Fluorolog 2 fluorometer equipped with a photon-counting detector. The temperature of the stirred cuvette was held at 20°C. After incubation with HPTS-containing apoB–liposome conjugates for different times, cells were washed twice with PBS-CMG and incubated for 5 min at 37°C with 1.5 ml PBS containing 3 mM EDTA. Cells were dislodged and diluted to $5 \cdot 10^5$ cells/ml in PBS. Peak heights were measured at 510 nm emission at the three excitation wavelengths 403, 413 and 450 nm. Fluorescence units are expressed as photon counts per second.

Fluorescence microscopy. After incubation with HPTS-containing apoB–liposome conjugates, cells were washed twice with 2 ml PBS-CMG and viewed with a Leitz fluorescense microscope. The pH-independent fluorescence of HPTS could be viewed with a filter set, which excites in a wide violet band (350–410 nm), or with a filter set having a narrow blue excitation band (450–490 nm), which visualizes the fluorescence of HPTS at neutral or basic pH.

Analytical procedures. Protein was measured by the modified Lowry method [14] or by using the Bio-Rad protein kit (Bio-Rad Laboratories, Richmond, CA, USA), with albumin as standard. Phospholipid concentration was determined by the method of Bartlett [15] or by measurement of [^{3}H]POPC by liquid scintillation. Radioactivity was measured in a Beckman LS-3801 scintillation counter after dissolving the samples in Liquiscint scintillation fluid.

Results

Preparation and characterization of apoB–liposome conjugates

The heptane extraction method for delipdation of LDL used in this study yields a product, which is devoid of neutral lipids but with the phospholipid content essentially intact [12]. In a set of preliminary experiments a panel of different conjugation methods were tested, including spontaneous conjugation, dehydration-rehydration and transient pH jump [16]. However, the method finally adopted, detergent solubilization and removal, proved to be superior to those methods. Two relatively mild 'biological' detergents; NaC and OG were chosen for the experiments. For the appropriate use of the detergent solubilization and removal conjugation method the amount of detergent to be added must be determined for each specific system. The concentration should be enough to totally solubilize the lipids in the presence of the protein, but unnecessary excess should be avoided. The amount of detergent to be added was determined by titration of liposomes containing a self-quencing concentration of carboxyfluorescein (CF, 90 mM) and apo B with the detergent in question. The detergent concentrations showing maximum release of encapsulated CF, corresponded to detergent/POPC ratios of 22 and 3 for OG and NaC, respectively. These values were applied in the conjugation experiments.

The solubilization and detergent removal method proved to to be a fast, convenient and effective method for the conjugation of apoB with POPC-cholesterol liposomes. An almost total (96%) recovery of both protein and phospholipid was noted after removal of detergents by adsorption on Bio-Beads SM-2. Fig. 1 shows the separation on a Sepharose CL-4B column of liposomes composed of POPC/cholesterol (2:1 molar ratio) conjugated with apoB in an initial ratio of 2:1 (weight ratio) between POPC and apoB. The effluent was analyzed for phospholipid and protein and in some experiments the elution profile of encapsulated CF was recorded. The results demonstrate an effective conjugation of apo B with the liposomes. The reproducible elution profiles show two peaks; a sharp peak near V_0 and a small diffuse peak near the V_t of the column. The first large peak represents conjugates, which was demonstrated by the coelution of entrapped CF with phospholipid and protein. Only traces of fluorescence was noted in the second small peak. The POPC/apoB weight ratios in the major peak were found to be very near that of the starting value of 2:1. The integrity of the conjugates was confirmed by Metrizamide gradient centrifugation resulting in a recovery of about 85% of apoB in cojugates. No difference was noted in yield for conjugates formed with native or acetylated apoB.

The analytical gel filtration indicated that the OG

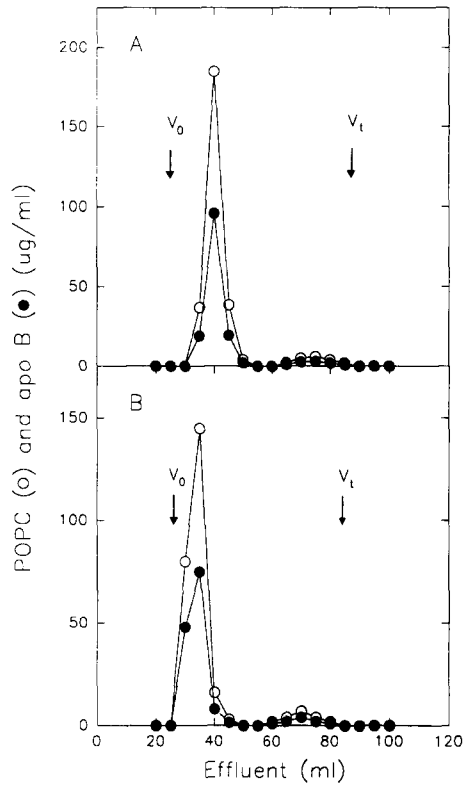

Fig. 1. Gel-filtration chromatography on a Sepharose CL-4B column (2.5×60 cm) of apoB–liposome conjugates prepared by the detergent solubilization and removal method as described in Materials and Methods. (A) Elution profile of conjugates prepared with sodium cholate (NaC) and (B) octyl glucoside (OG) as detergent. Arrows mark the void (V_0) and total (V_t) volumes of the column; (○) μg of POPC and (●) μg of apo B per ml of effluent.

conjugates were somewhat larger than those prepared with NaC. This observation was confirmed by the quasi-elastic light-scattering measurements. The conjugation of liposomes with apoB increased the diameter to some degree giving average values of 222 and 140 nm for OG and NaC conjugates, respectively, and 141 and 92 nm for corresponding liposomes, prepared by the same methods. At sucrose density gradient ultracentrifugation the apoB–liposome conjugates appeared as a distinct band with peak maximum at $d = 1.07$ g/ml. The phospholipid and protein bands overlapped completely. The measurements of the CF leakage showed a somewhat increased leakage for the conjugates compared to corresponding liposomes, giving values of 1.5 and 2.4% per day for OG and NaC conjugates and 1.5 and 0.9% for corresponding liposomes, respectively.

Biological activity

The spectral shifts of HPTS with changes in pH make it a useful marker of the fate of encapsulated dye following endocytosis [17,18]. HPTS exhibits two major fluorescence excitation maxima (403 and 450 nm) with complementary pH-dependence in the range 5–9. The peak at 403 nm is maximal at low pH values while the peak at 450 nm is maximal at high pH values. The cellular fate of the dye encapsulated in apoB–liposome conjugates and unconjugated liposomes was followed by fluorescence microscopy and fluorometry. At microscopy the uptake into acidifying compartments was followed by use of an essentially pH-insensitive violet filter set (350–410 nm) and a blue filter set (450–490 nm) which excites the pH-sensitive 450 nm peak. After a short time of incubation (< 0.5 h) with conjugates and liposomes, punctate fluorescence was seen on the cell surface, as well as in intracellular compartments under both short and long-wavelength excitation. At later times perinuclear vacuoles appear, with strong fluorescence at 350–410 nm illumination, but with much weaker fluorescence when excited with long wavelengths. Cells incubated with conjugates showed a more intense fluorescence than those incubated with liposomes. This fact was especially obvious for cells incubated with the OG conjugates, which after 3 h incubations showed a massive accumulation of dye in large vacuoles visible under violet, but not under under blue illumination (Fig. 2). These large-diameter acidic punctates may result from leakage of encapsulated dye in endosomes and lysosomes. The accumulation of dye was more accenturated for CV-1 than for J774 cells and much so in the large acidic vacuoles.

The cellular uptake of liposomal phospholipid can be calculated starting with the measured fluorescence values and the known values for fluorescence per nmol liposome or conjugate phospholipid. The quantitative measurements of POPC incorporation into cells (Fig. 3) confirmed the observations made by fluorescence microscopy. NaC conjugates showed a clear but only moderate predominance in dye incorporation com-

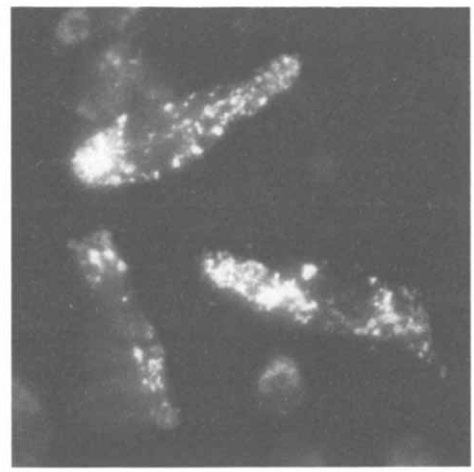

Fig. 2. Fluorescence micrographs of J774 macrophages treated with HPTS-containing aopB-liposome conjugates. The cells were treated with the preparation for 3 h at 37°C, washed, and viewed with a water immersion objective by epifluorescence with λ_{ex} 350–410 filters.

pared to corresponding liposomes. OG conjugates on the other hand gave a 5–6-fold increase in the rate of uptake compared to liposomes. Calculated on the basis of cell number the uptake of conjugates and liposomes was about ten times larger for CV-1 than for J774 cells. The observation by microscopy that much of the fluorescence gradually accumulated in acidic intracellular compartments was also confirmed by calculations of the 450/413 nm ratios. The OG conjugates gave ratios of 0.33 and 0.41 for CV-1 and J774 cells, respectively, after 3 h of incubation. These values correspond to pH values of 6.1 and 6.3, respectively, as estimated from the pH calibration curve of HPTS [18]. Thus, most of the dye endocytosed after 3 h of incubation resides within endosomes and lysosomes at low pH. The corresponding 450/413 nm ratios for OG liposomes were 0.52 and 0.71. NaC conjugates and liposomes gave similar values around 0.70 with both types of cells.

The initial studies clearly showed a preferential cellular uptake of OG conjugates. The further experiments were thus concentrated on this type of preparations. The internalization process was further studied

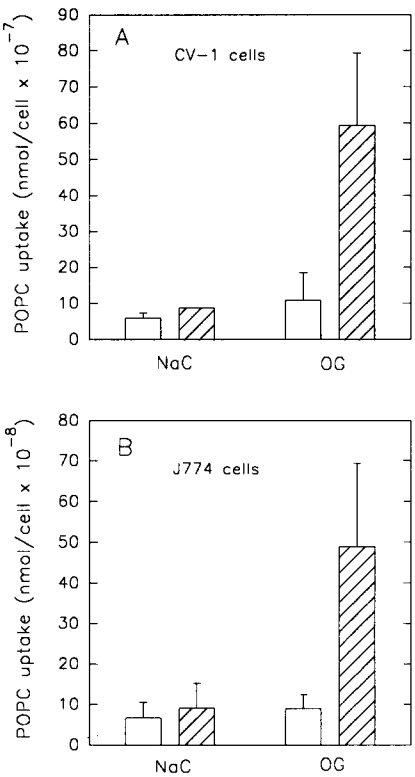

Fig. 3. The cellular uptake of HPTS-containing liposomes (open bars) and apoB–liposome conjugates (hatched bars) expressed as nmol POPC per cell. CV-1 (A) and J774 (B) cells were exposed to preparations prepared with NaC (bars to the left) and OG (bars to the right) as detergents at a concentration of 80 nmol of POPC per ml PBS for 3 h at 37°C. After incubation cells were washed with PBS and the cell-associated fluorescence was measured and the corresponding POPC values calculated.

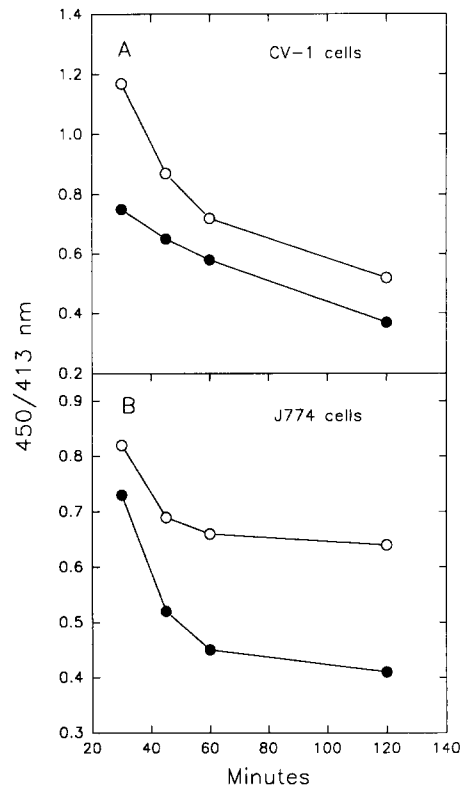

Fig. 4. Internalization into low-pH compartments of HPTS-containing liposomes (○), and apoB–liposome conjugates (●) by (A) CV-1, and (B) J774 cells in culture. The preparations were made with OG as detergent. The cells were incubated with respective preparation for 30 min, then washed with PBS and the incubation proceeded for different lenghts of time. The uptake process was followed by measurements of the 450/413 nm excitation wavelength ratio.

by exposition of cells to the conjugates for 30 min and then they were washed carefully and incubated for different times in PBS. Removal of free liposomes or conjugates resulted in a moderate reduction of total cell-associated HPTS with time at excitation wavelengths of 403 and 413 nm (data not shown). The peaks at 450 nm showed a more dramatic change and decreased progressively and with a faster rate for the conjugates than for the liposomes. The normalized 450 nm values (450/413 nm ratios) from CV-1 and J774 cells incubated with liposomes and conjugates are shown in Fig. 4. The fast decrease in 450/413 ratios noted for both cell types and both preparations is consistent with the expected change due to the acidification of HPTS. This process is somewhat faster for the J774 cells than for the CV-1 cells and becomes largely complete 30 min after removal of the conjugates.

The concentration-dependent uptake of encapsulated dye is shown in Fig. 5. At low concentration, there is an marked increase in uptake with increase in concentration of conjugates, which may in large part be due to binding of the particles to the cell surface. For

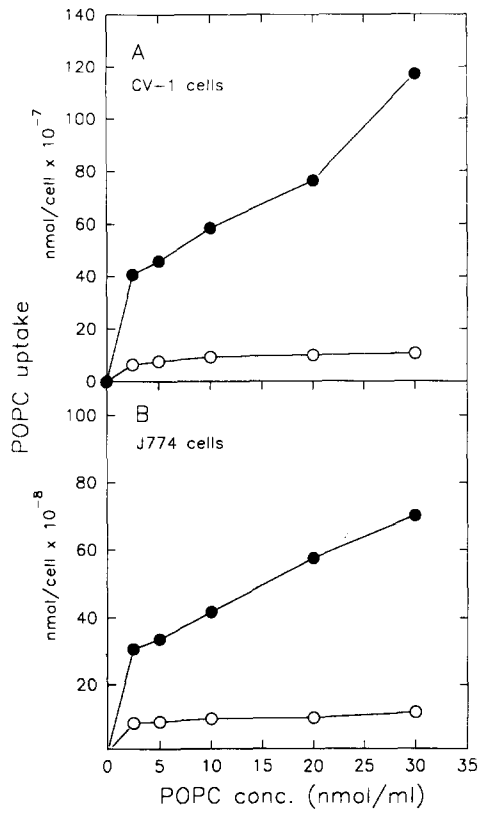

Fig. 5. The concentration-dependent uptake of HPTS-containing liposomes (○) and apoB–liposome conjugates (●) by (A) CV-1 and (B) J774 cells in culture. OG was used as detergent. The amounts of the preparations taken up by cells were calculated from measured values of HPTS fluorescence counts per nmol POPC.

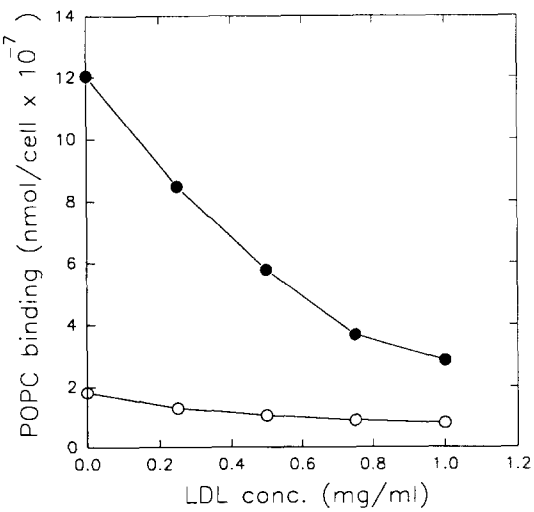

Fig. 6. The ability of native LDL to compete with HPTS-containing liposomes (○) and apo B-liposome conjugates (●) for binding to CV-1 cells in culture. The preparations used were made with OG as detergent. In each sample the final concentration of POPC was 80 nmol/ml and that of LDL as indicated. After incubation for 1 h at 4°C the cells were washed with PBS and the cell-associated fluorescence measured and the corresponding POPC values calculated.

the conjugates the uptake is dependent on the concentration, while the uptake of liposomes was less sensitive to the concentration.

The specificity of the binding of apoB–liposome conjugates to cells was tested by the ability of native LDL to compete with OG conjugates (and liposomes) for the binding to the LDL receptors on CV-1 cells. The addition of LDL to the incubation mixture decreased the binding of conjugates in a concentration-dependent and reproducible way (Fig. 6), demonstrating a competitive binding to the LDL receptor. The specificity of the binding of the conjugates to the cells was further confirmed by displacement experiments with high concentrations of HDL and albumin (1 mg/ml). No significant reduction of conjugate binding was noted. On the other hand, when the incubation was performed in the presence of heparin (3 mg/ml), which is known to displace LDL from the receptor [5], the binding of the apoB liposome conjugates to the cells was abolished. It was also noted that the binding of liposomes to cells was to some degree hindered by LDL (Fig. 6).

Discussion

The LDL receptor pathway has been studied extensively and is known in many details [5]. The use of this pathway to deliver cytotoxic agents to cancer cells has been proposed by several authors [4,19]. In a previous paper, the successful targeting of a lipophilic antineoplastic drug to cancer cells in culture was performed [20]. In the present study the targeting capability of apoB is combined with the capacity of liposomes to encapsulate water-soluble compounds. Drug targeting via the LDL receptor pathway is feasible by means of apoB–liposome conjugates as long as the coupling reaction preserves liposome integrity, as well as the binding properties of apoB to the receptor. Considering the dimensions and complexity of the apoB molecule (apparent molecular mass 550 kDa [21]), the fullfillment of these requirements might seem all but certain. However, the detergent solubilization and removal method proved to work excellent for conjugation of apoB to liposomes. In a fairly short time a stable product was produced with a very good yield. The preparations made by OG solubilization proved to have a good biological activity. The competitive inhibition of the cellular binding of conjugates by native LDL strongly indicates a receptor-mediated uptake. The LDL concentrations needed for displacement of conjugates were higher than those valid for fibroblasts [22], but similar to those obtained with leukemic lymphocytes [23]. The specificity of the binding of apoB–liposome conjugates to CV-1 cells was further confirmed by the lack of competition with HDL and albumin and the displacement by heparin. The experiment with the pH-sensitive

probe HPTS, revealing a cellular uptake to an acid milieu, was also cosistent with a LDL receptor uptake via the endosome-lysosome pathway. It is interesting to note that also the binding of liposomes to some degree was inhibited by LDL. This observation supports the hypothesis that liposomes can enter cells through the coated pit/coated vesicle system [24]. The poor biological activity of the NaC-conjugates is not easily explained and this study offers no answer to this problem.

Special aspects of targeting with apoB are offered by the possibility to chemically modify the protein and thereby change its fate in vivo. The acetylation used in this study leads to modification of the lysine residues of the protein, which results in a more negatively-charged particle [9]. ApoB modified in this way is specifically recognized by the scavenger receptor on macrophages and endothelial cells [7]. The uptake of liposomes by the macrophage cell type J774 was enhanced by conjugation to acetylated apoB. This fact supports the presumption that the conjugates are taken up by the scavenger receptor. Like uptake via the LDL receptor, internalization via the scavenger receptor proceeds through coated vesicles and, finally, degradation in lysosomes [25]. Another possibility to modify apoB is by lactosylation, which induces rapid clearance due to galactose-specific uptake by the liver [7].

The pH-sensitive and membrane-impermeant fluorescence dye HPTS proved to be a very useful tool to study the kinetics of endocytosis. Endocytosis is detected by the large pH-dependent shift in excitation wavelength. As a fluorescence indicator useful for both microscopic and fluorometric analysis, HPTS provides several advantages; good water solubility and photostability, a pK_a value in the physiological range and a high fluorescence quantum yield, and has an isosbestic point at 413 nm, permitting corrections of the measurements to the total amount of dye present in the sample. More detailed descriptions of the properties and use of HPTS have been presented in recent papers [17,18].

The intracellular fate of the conjugates might present a problem. The results of this study shows unequivocally that they end up in acidic compartments like endosomes and lysosomes. As long as a lysosomal route is aimed at this fact is favourable, but often enough this is not the case. However, a special type of liposomes, pH-sensitive liposomes, have been developed to circumvent delivery to the lysosome [26]. The appropriately designed pH-sensitive liposome should transfer its content into the cytoplasm. Such a behaviour is important, e.g., for delivery of DNA to cells, which could be one application of apoB–liposome conjugates.

It can be concluded that apoB- and modified apoB–liposome conjugates obviously have a potential use as effective targeting systems for bioactive agents in vitro. However, their large scale use for drug targeting in vivo might be more problematic. The utility of liposomes for delivery of therapeutic agents was for a long time hampered by the rapid recognition and removal of these carrier particles by cells of the mononuclear phagocytic system (MPS). Recently, however, sterically-stabilized liposomes with long circulation half-lives have been described [27]. Such liposomes contain lipids with special headsgroups such as GM_1, PI, PEG-PE, which contribute to their long blood residence time. Liposomes made up of phosphatidylcholine and cholesterol were considered appropriate for this in-vitro study, but it seems plausible that apoB can be complexed to sterically stabilized liposomes as well. The method for delipidation of apoB applied in this study, involving stabilization of the protein structure by sucrose, was chosen because this procedure preserves the clearance rate of the apoprotein [11]. By the extraction with cold heptane the neutral lipids, but not the phospholipids are removed from LDL [12]. The phospholipids apparently help to stabilize the structure of apoB. The conformation of apoB in LDL is an elongated form that wraps around the LDL particle and interacts with the polar surface with many but short anchoring points [28]. In fact, the surfaces of LDL and liposomes with their surface monolayer of phospholipid and cholesterol offer a similar environment for apoB–lipid interactions. Recent structural studies of apoB have confirmed that it retains its native conformation by recombination with lipid after a mild delipidation [29].

A very important question regarding the feasibility of liposomes as drug carriers is whether they are able to cross the anatomical barriers, such as the capillary wall, which separates the tumor cells from the bloodstream. Targeting of liposomes will be successful only if they are able to exit from the circulation and, thus, gain access to target cells in the extravascular compartments. In this context, the anatomy of the microcirculation can be expected to be of crucial importance. Blood capillaries are classified into three different groups; continous, fenestrated and sinusoidal capillaries. In contrast to the continous and fenestrated capillaries the sinusoidal ones offer relatively large gaps for the liposomes to peneterate the endothelium. A factor, which might favour the access of liposomes to tumors is the increased permeability of tumor vasculature compared to normal tissue [30]. In fact, it seems likely that the ability of some liposome formulations to accumulate in transplantable human tumors, at least in part, is due to increased microvascular permeability [31].

ApoB–liposome conjugates meet many requirements for a good targeted delivery system; all components are biocompatible, biodegradable and nontoxic. LDL/drug complexes, which resemble apoB–liposome

conjugates in many respects, have proven to be effective drug carriers in vitro (for review, see Ref. 19). In recent studies an increased therapeutic effect was obtained through use of LDL as a carrier administered in vivo to both animals [32,33] and humans [34]. These results support the view that also apoB–liposome conjugates may prove to be useful for drug targeting. However, it should be stressed that it is not possible to obtain absolute selectivity, since normal cells also have LDL receptors. This problem can partly be solved by down-regulating the LDL receptors in the liver and the adrenals [35] and, since most antineoplastic drugs in use today are totally untargeted, even partial success would be an appreciable improvement.

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