Surface Charge and the Association of Liposomes with Colon Carcinoma Cells

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Colon Cancer Cells, Charged Liposomes, Electrostatic Surface Charge

Interaction between the plasma membrane and aggregate lipid surface determines how efficiently the encapsulated drug will be delivered into the cell. Electrostatic interactions are one of the main forces affecting liposome and aggregate association with the charged cell surface. In this study, the effect of surface charge on the association of liposomes with human colon CX-1.1 cancer cells was studied. When phosphatidylserine was incorporated into a lipid bilayer, the amount of liposomes associated with cells tended to increase along with the amount of negatively charged lipid present in the liposomal lipid bilayer. When the cationic lipid dioleoyl-1,2-diacyl-3-trimethylammonium-propane (DOTAP) was included into the liposome formula, their uptake by the cells was also increased. Maximum binding occurred when the amount of positively charged lipids in liposomes was about 10 mol% of lipids.

Introduction

Lipids are becoming a common constituent of various supramolecular ensembles that serve as drug delivery systems. They are liposome building material when the latter are used as small compound carriers, and form complex aggregates with large macromolecules like DNA and proteins (Allen, 1998; Langner, 2000; Langner and Kral, 1999; Lasic, 1998; Lasic and Templeton, 1996; Miller, 1998; Sharma and Sharma, 1997; Smith et al., 1993). Regardless of their structure, it is advantageous to obtain aggregates with the outer surface formed by a lipid monolayer or bilayer that protects the included material from degradation. Such a surface also determines how the entire supramolecular structure will interact with soluble proteins and cell membranes. A number of lipid surface properties relevant to such interactions have been identified, i.e. electrostatic surface charge, hydrophobicity, steric constrains and the packing of lipid molecules forming the surface.

Electrostatic forces participate in essentially all intermolecular interactions, since the majority of physiologically relevant molecules carry residual charges. Therefore, electrostatics are among the major driving forces of supramolecular aggregate formation. Lipid surface electrostatic charges not only influence the membrane formation processes but also alter the properties of the adjacent aqueous phase and determine how the entire aggregate will interact with body fluid components, in turn affecting the aggregate stability (Cevc, 1990; Duzgunes and Nir, 1999; Langner and Kubica, 1999; Miller, 1998; Miller et al., 1998; Smith et al., 1993; Zuidam and Barenholz, 1998).

Studying the association of the supramolecular aggregate with the cell surface, it is difficult to distinguish between the involvement of electrostatic forces and other types of interactions relevant to the process. The formation of lipid/DNA complexes is a good example of such a situation. There are numerous experimental data showing that the lipid/DNA aggregate should carry a net positive charge in order to efficiently associate with the negatively charged cell plasma membrane, which presumably ensures its subsequent uptake by the cell (Chen et al., 2000; Zhang et al., 1999). Such observations, nevertheless, have not been confirmed by experiments in vivo, where negatively charged aggregates are frequently better transfection agents (Delepine et al., 2000; Floch et al., 2000). The difficulties to explain such contradictory result is mainly due to lack of knowledge of...
lipid/DNA aggregate topology and properties, and its interaction with biological structures. In such a case, the major difficulty is to separate the effect of electrostatic interactions from other factors, i.e. lipid propensity to form non-lamellar structures, lateral lipid separation, steric constrains, aggregate size and possible interaction of the DNA molecule with the cell surface (Delepine et al., 2000; Hui et al., 1996; Kennedy et al., 2000; Zuidam and Barenholz, 1998). For this reason, we have used liposomes whose outer surface, when charged, can serve as a simplified model of aggregate interaction with the biological membrane.

Aggregate association with cells depends on forces acting between the continuous outer lipid surface and plasma membrane, which depend rather on local than global aggregate properties at the contact area. Following this argument, we have assumed that the liposomal outer surface can serve as a general model that mimics interaction between cell surface and the aggregate with a continuous lipid surface. In this paper, we present a systematic study of the effect of liposome net surface charge on its association with human colon cancer cells (CX-1.1). In addition, the role of exposure time and the presence of bovine serum albumin in the culture on uptake of various liposome formulations has been studied.

Materials and Methods

Materials

Egg-phosphatidylcholine (Egg-PC), bovine brain phosphatidylserine (PS), dioleoyl-1,2-diacyl-3-trimethylammonium-propane (DOTAP) and rhodamine-PE were purchased from Avanti Polar Lipids Inc. (Alabaster, AL USA).

Liposome formation and characterization

Lipids were mixed in chloroform with an appropriate amount of Rhodamine-PE (1 mol%). The chloroform was then removed under vacuum. Phosphate buffered saline (PBS), with or without 10% fetal bovine serum (FBS), was added and the sample vortexed, followed by brief sonication in a bath sonicator. The amount of the fluorescent probe in the lipid bilayer (1 mol%) was low enough to disregard the influence of self-quenching. When liposomes were exposed to serum extensive aggregation was observed, but the fluorescence intensity was not drastically reduced (when corrected for light scattering). This shows that close vesicle proximity does not interfere with probe fluorescence. Vesicle size was determined using dynamic light scattering (ZETASIZER 5000 analyzer, Malvern Instruments, Worcs, UK).

Cell line and incubation conditions

A human colon cancer cell line CX1.1 was obtained from Deutsche Krebsforschungszentrum (Heidelberg, Germany). Cells were cultured in an a-minimal essential medium, supplemented with a 10% fetal bovine serum inactivated by heat (FBS, Gibco BRL, Grand Island, NY, USA), 2 mM L-glutamine, 100 units/ml penicillin G, 100 mg/ml streptomycin. They were maintained at 37 °C in 5% CO2/humidified air. The cultured medium was removed and liposomes, suspended in the appropriate medium, were added. Cells were incubated for the desired period and liposomes that remained in the suspension were washed away. The amount of lipid in all samples was 0.67 mmol/ per well.

Flow cytometry

The cells were detached by 0.05% trypsin/0.02% EDTA, washed and resuspended in 50 mM Tris/HCl pH 7.8, 180 mM NaCl and 18 mM MgSO4, containing 1% BSA (Sigma, St. Louis, USA) and 0.1% NaN3. They were subjected to fluorescence analysis using FACSscan (Becton Dickinson). The sample was excited with laser beam (488 nm) and the emitted light was passed through the multiband DAPI-FITC-Rhodamine filter cube before fluorescence intensity was measured. Five thousand randomly selected cells from each sample were measured and analyzed. Data were processed and mean fluorescence intensity was calculated using WinMDI 2.8.

Results

Liposome characterization

Liposomes interact with polions and serum proteins present in the culture medium, which causes their aggregation and precipitation (Chonn et al., 1992; Gao and Huang, 1991; Leckband et al., 1993; van-de-Wetering et al., 1999; Yang and Huang, 1997). As such interactions depend on li-
posome lipid composition, liposomes of different formulas were incubated with or without 10% FBS, and their sizes measured shortly after sample preparation. It was found that liposomes formed by phosphatidylcholine and its mixtures with DOTAP and/or phosphatidylserine at low concentrations, had a broad size distribution when measured by dynamic light scattering (mean sizes about 1000 nm). Marked aggregation was observed only with vesicles formed by pure PS (even in the absence of fetal bovine serum), and those containing a large fraction of DOTAP in the presence of serum.

The effect of liposome surface charge on their interactions with colon carcinoma CX1.1 cells

Liposomal bilayer properties vary with lipid composition. Some lipids that form liposomes may carry an electric charge, others are prone to conformational transformation or form bilayers that differ in their molecular organization. In order to avoid the interference of factors other than surface electrostatic charge, lipid composition was selected in such a way that the lipid bilayer was in a liquid crystal state. Egg phosphatidylcholine, bovine brain phosphatidylserine and DOTAP, used to form liposomes, are known to remain in the Lα fluid phase at physiological conditions and are far from any phase-transition temperature that would affect the molecular organization of their liposomal membranes (Koynova and Caffrey, 1998; Marsh, 1990). For the same reason, cholesterol was not included in the preparation: when mixed with other lipids, it may induce the lateral non-uniformity of the lipid bilayer, potentially introducing additional factors (other than electrostatic forces) to liposome interaction with cells (Radhakrishnan and McConnell, 1999; Raffy and Teissie, 1999; Silvius et al., 1996).

Negatively charged liposomes

Figure 1A shows the effect of negatively charged lipid (phosphatidylserine) on liposome association with cells after 24 h of incubation when mixed in different ratios with egg-PC. Fluorescence intensity associated with cells increased together with the amount of PS present in the liposomal bilayer, and that increase was more than an order of magnitude when the cells were incubated with pure phosphatidylserine liposomes, as compared to those containing only egg-PC (Fig. 1A). The extensive uptake of PS-liposomes may be a combined effect of their aggregation and high surface charge density.

Figures 2A and 2B show the flow cytometric analysis of CX-1.1 cells incubated with egg-PC liposomes for 5 h and 24 h, in the presence and absence of 10% FBS. As expected, the amount of liposomes associated with cells increased with incubation time; surprisingly, fluorescence intensity was noticeably higher after 24 hours if colon cancer cells were incubated with neutral liposomes in the presence of FBS. Presently, it is impossible to say if the presence of fetal bovine serum directly affects liposomes interactions with the plasma membrane or if its presence improves cell vitality, which increases liposome intake. There was no difference in the viability of cells grown in the presence and absence of FBS.

As in the case of neutral liposomes, the intake of negatively charged liposomes by CX-1.1 cells increased with the incubation times. However, in contrast to PC-liposomes, the presence of 10% FBS had only limited effect on the intake efficiency after 24 hours of incubation (Figures 2C and 2D).
Positively charged liposomes

When positive charge was introduced onto the vesicle surface by mixing DOTAP with egg-PC, the effect of its density on liposome uptake efficiency was quite different from that of negatively charged liposomes (Fig. 1B). An increase in DOTAP concentration in the lipid bilayer of up to 10 mol% was associated with higher liposome uptake by cells, however, further increase of DOTAP content reduced their binding by CX-1.1 cells monotonically. When cells were incubated for 24 hours with liposomes containing 10 mol% of DOTAP, their fluorescence increased almost by an order of magnitude compared to that of liposomes containing pure egg-PC. However, when DOTAP content was increased to 50 mol%, the amount of fluorescence associated with cells fell to half that of liposomes containing 10 mol% DOTAP. Figures 2E and 2F show the effect of incubation time and fetal bovine serum presence on the cellular intake of liposomes containing 10 mol% of DOTAP. Similarly as has been shown for neutral and negatively charged liposomes, the intake of positively charged liposomes increased following longer incubation time (24 hours). However, in contrast to previous results, the presence of 10% FBS in the culture medium reduced positively charged liposome uptake to a certain extent.

Discussion

In this paper three parameters relevant to liposome intake by cells were examined, i.e. surface electrostatic charge, incubation time and serum...
presence. The introduction of electrostatic charge of either sign onto the liposome surface enhances its association with colon cancer cells. It has been shown previously that in the case of the lipid-DNA aggregate, surface positive charge enhances its association with a negatively charged cell surface in vitro (Duzgunes and Nir, 1999). On the other hand, negative charge is believed to stimulate liposome uptake in vivo, which is likely due to interaction with serum proteins (Chonn et al., 1992). However, it has never been shown before that liposome association with a single cell type in vitro is enhanced by the presence of both negative and positive charge. Such a result suggests that factors other than surface electrostatics govern liposome association with colon carcinoma cells, or that various mechanisms lead to the association and subsequent intake of liposomes. This dilemma awaits further studies. The existing difference between influence of surface charge density on the efficiency of liposome association with cells justifies the assumption that DOTAP and PS affect cells in a different manner. It has been shown that cationic lipids have a toxic effect on cells in a variety of ways, e.g. by inducing apoptosis (Aramaki et al., 1999), causing oxygen radical-mediated damage (Dokka et al., 2000) and disturbing plasma membrane structure (Urmonet et al., 1998). Hence, excess cationic lipid accumulated by the cell may obstruct the extensive intake of liposomes. Toxicity is a likely reason why liposomes that contain a high fraction of DOTAP do not associate with CX-1.1 cells as efficiently as with those containing 10 mol% of this lipid. Such an effect is not expected when liposomes contain phosphatidylcholine and/or phosphatidylserine, as these lipids are not toxic. Accordingly, it has been shown that exogenous phosphatidylserine stimulates endocytosis, therefore liposomes containing the highest amount of this lipid are likely to associate with cells most efficiently (Farge, 1995; Farge et al., 1999).

Exposure time to liposomes affects their association efficiency with cells in a predictable way, i.e. the longer the incubation time, the more efficient the process and pronounced the differences between different liposome formulations.

The role of serum in interfering liposome association with the cell plasma membrane is, according to literature, a complex process that depends predominantly on liposome lipid composition and formulation (Chonn et al., 1992; Yang and Huang, 1997). Our data imply that the addition of serum alone to the incubation medium has only a limited effect on the association of negatively and positively charged liposomes with colon carcinoma cells. In our hands, the presence of serum did not prevent liposome uptake, but rather modestly enhanced it, and its effect was evident only after extended exposure (24 h).

In this paper we have shown that interaction between the cell surface and liposome surface is altered when either negative and positive charges are present. For this reason, electrostatic interactions alone cannot be used as an indicator of when liposome composition is optimized with regard to its association with the cell. Other liposome properties, such as its propensity to form non-lamellar phases, opsonization extent, and association with selected membrane components (receptors, the glycocalix) are likely to be equally important. Furthermore, subsequent liposome internalization may depend on liposome component toxicity and/or stimulating activity.

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