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ORIGINAL PAPER

Efficiency of cytoplasmic delivery by non-cationic liposomes to cells in vitro: A confocal laser scanning microscopy study

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Abstract It is necessary to understand liposomal uptake mechanisms and intracellular distribution in order to design more efficient gene (drug) carrier systems. Until now, a few studies have been carried out using confocal laser scanning microscopy (CLSM) to investigate the cellular uptake and transfection mediated with liposomes. So, by CLSM, we demonstrated that artificial virus-like envelope (AVE) vesicles labeled with rhodamine-PE (Rh-PE), carbocyanine (DiI) and carboxyfluorescein (CF) were investigated into the cytoplasm of two human cell lines, Mewo (human melanoma cell line) and HepG2 (human hepatoma cell line) cells grown in DMEM medium supplemented with different percentages (0%, 30%, and 100%) fetal calf serum (FCS). The liposome uptake was dependent on the cell line, in view that the whole process of liposomes associated with cells (uptake) is a two-step process involving binding and endocytosis. Based upon the various assays used to measure cellular uptake of liposomes, we conclude the efficacy of cytoplasmic delivery by AVE-liposomes to cells in culture.

Introduction

Success of human gene therapy depends upon the development of delivery vehicles or vectors, which can selectively deliver therapeutic genes to target cells with efficiency and safety. The ideal vehicle should avoid immediate uptake by the mononuclear phagocyte system and have prolonged circulation in blood, thus increasing the probability of reaching the desired targets. In addition, the vehicle should be able to deliver its contents efficiently into the cell cytoplasm, avoiding lysosomal degradation [23]. The most widely used types of vehicles for gene delivery are: viral

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(e.g., adenovirus, retrovirus and adeno-associated virus) and non-viral (e.g., liposomes, polymer, and peptides) [13]. Viral vectors are often highly efficient, but safety and immunogenicity are issues of potential concern, and the limited transgene size often possesses a serious obstacle [19]. Non-viral vectors, on the other hand, frequently face the problem of low transduction efficiency. A large number of cationic lipids have been developed to improve the gene transfer, but toxicity, lack of biodegradability and low transfection efficacy still remain as problems [6]. PH-sensitive anionic liposomes have also been shown to mediate gene transfer but suffer from poor encapsulation efficiency due to the large size and the negative charge of the uncondensed DNA [24].

Recent studies have shown an efficient, systemic transgene expression in many cell lines (in vitro) by using an anionic liposomal vector, based on the composition of retroviral envelopes (artificial virus-like envelopes, AVEs) [9,19,20,5,17,12]. AVEs mimic the lipid composition of retroviruses [1]. These natural lipids are anionic, in contrast to their artificial cationic counterparts, interact only weakly with their biological environment, and, therefore, are non-toxic [19]. These AVE-liposomes have special fusogenic properties, allowing them to transport encapsulated or associated drugs into cells. Recently, nuclear localization of oligonucleotides (ON) could be observed with the AVE formulation and it can be targeted to special cell types using receptor-sensitive ligands, thereby enhancing localized uptake of ON in the cell of interest [9,25]. Müller et al. [19] showed that 99% of HUVEC endothelial cells are transfected with AVE/PEI/DNA complexes coupled with a cyclic RGD-peptide as a targeting device for the $a_v\,\beta_3$-integrin on tumor endothelial cells.

Despite the enormous amount of work on the use of liposomes for drug delivery, the molecular mechanisms of liposome-cell interactions have not been elucidated completely [4]. The interaction of liposomes with cultured cells in vitro is complex. It involves surface binding, internalization and possible release of liposomal contents. Cellular uptake of liposomes has a varying dependence on the liposome composition, charge type, size and on the cell line [14].

It is necessary to understand liposomal uptake mechanisms and intracellular distribution in order to design more efficient gene (drug) carrier systems. The interaction of phospholipids vesicles (liposomes) with cells is a promising area in cell biology since it holds the possibility of introducing new material into the cytoplasm and plasma membrane of the cell. The use of fluorescence microphotometry yielded information on the relative level of association of various vesicles to various cells but was not used to obtain an estimate of the absolute amount of vesicle lipid associated with the cell.

Until now, a few studies have been carried out using confocal laser scanning microscopy (CLSM) to investigate the cellular uptake mediated with liposomes. Confocal microscopy is a well-established technique for the investigation of three-dimensional structures in biological and industrial materials. The basis of this success is the optical sectioning capability of this type of microscopy, which enables one to study 3D-structure of intact specimens in their natural environment. CLSM has the major advantages that it yields a very short depth of focus, its transverse definition and the contrast of the image are better than a standard microscope, the device is very well-suited for optical cross-sectioning, and with the use of a laser beam, the intensity of illumination can be very high. With CLSM one can slice incredibly clean, thin optical sections out of thick fluorescent specimens; view specimens in planes running parallel to the line of sight; penetrate deep into light-scattering tissues; gain impressive three-dimensional views at very high resolution; and improve the precision of microphotometry. In order to study the intracellular disposition of liposomes, our approach was to improve the detection by CLSM. It has been used to provide a qualitative view of the intra- and intercellular heterogeneity of liposome uptake.

The interaction between liposomes and blood components is the first step after the administration of liposomes, and it is the interaction, which determines the fate of liposome disposition in the body. Since serum components affect the uptake of liposomes, it is important to perform in vitro experiments in the presence of relatively high concentrations of serum before attempting to derive conclusions regarding the mechanisms of liposome-cell interactions in vivo [4]. Serum components such as antibodies, complement, or fibronectin—opsonins are known to enhance liposome uptake by the reticuloendothelial system (RES) such as macrophages in the liver and spleen [26]. The presence of serum often reduces the transfection efficiency of liposomal vectors [8,7,10,16]. This may be due to the premature release of DNA from the complexes or its degradation by the nucleases.

Our first aim was to find an answer to some crucial questions: are Mewo, and/or HepG2 cells capable of taking up AVE-liposomes? Does this uptake, if occurring, consist of mere adsorption of intact vesicles to the cell surface, or are vesicles internalized within the cells? Furthermore, if internalization can be demonstrated, what is the mechanism of uptake?

In this work, we used CLSM to investigate the efficacy of cytoplasmic delivery of AVE-liposomes labeled with Rh-PE, DiI and carboxyfluorescein (CF) to two different human cell lines, Mewo (human melanoma cell line) and HepG2 (human hepatoma cell line) cells grown in DMEM medium supplemented with different percentages (0%, 30%, and 100%) of FCS.

Materials and methods

Materials

1,2-Dilauroyl-*sn*-glycero-3-phosphoethanolamine (DLPE), dioleoyl phosphatidyl serine (DOPS), were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Tris ultra pure buffer was obtained from (ICN Biomedicals Inc., Ohio, USA). Cholesterol (CHOL) was obtained from Calbiochem (La Jolla, CA, USA). 4(5)-carboxyfluorescein (CF) was purchased from Fluka (Germany).

Cell culture

Fetal calf serum (FCS), L-Glutamine (200 nM solution), Penicillin 5000 units/streptomycin 5000 mg, Trypsin 200 mg/l

and DMEM (Dulbecco's modified Eagle's medium) were obtained from Biowhittaker Europe, Verviers, Belgium.

Lissamine™-rhodamineB-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, triethyl-ammonium salt (Rh-DOPE) and DiI (1,1′-dioctadecyl-3,3,3′, 3′-tetramethylindocarbocyanine perchlorate) were from Molecular Probes (Eugene, OR, USA). All other reagents were of analytical reagent grade. The formulation of AVE was a gift of H. Schreier Consulting, Sebastopol, CA, USA. Mewo and HepG2 cells were obtained from Institute of Molecular Biology and Tumor Research, Philipps-University, Marburg, Germany.

Methods

Preparation of liposomes

AVE-liposomes are composed of equimolar amounts of DLPE, DOPS and CHOL [25]. Liposomes were formulated according to well-established methods of extrusion [21]. In short, the appropriate phospholipid composition was mixed in organic solvent in a 50 ml-round flask. The organic solvent was evaporated to dryness by a mini-rotavapor (Büchi, Switzerland). The resulting thin film was hydrated with tris buffer saline (10 mM tris, 140 mM NaCl, and pH 7.4) containing 100 mM carboxyfluorescein (CF). The resulting lipid suspension was extruded through 200 nm polycarbonate membranes (Nucleopore GmbH, Germany), using a commercially available extruder LiposoFast, Avestin Inc., Canada). Free CF was removed by dialysis (Mini Lipoprep, Dianorm GmbH, Germany) in Tris buffered saline containing 10 mM Tris and 140 mM NaCl at pH 7.4 after extrusion [26]. Size measurement was done by dynamic laser light scattering (Zetasizer 3000 HS, Malvern Instruments, Germany) and the size was in the range of 200 ± 30 nm. We labeled the liposomes with Rh-PE (0.2 mole%) or DiI (10 μg/ml DiI/2.5 mg/ml lipid).

CLSM

To investigate the uptake by CLSM, the cells were seeded at a density of 1×10^5 on 6-well plates. Mewo and HepG2 cells were grown on glass cover slips in DMEM medium supplemented with different percentages of FCS (0%, 30%, and 100% FCS), 1% glutamine and 1% penicillin-streptomycin solutions. The cells were washed away by rinsing three times with cold PBS (phosphate buffer saline). Cells were fixed with 4% PFA (paraformaldehyde) and cells were incubated with 0.1% sodium Borohydride buffer. Turn the coverslip containing the cells on a Mowiol (Colbiochem, Schwalbach, Germany) drop on a glass slide and examine with CLSM. Confocal microscopy was performed with True confocal Scanner, Leica TCS 4D and Leitz DM RXE upright microscope (Leica Microsystem Heidelberg GmbH) with a Krypton/Argon laser. The filter was OG 590 for DiI and Rh-PE.

Images were converted to TIF-format with Scanware 5.1 program. All optical sections were recorded with the same laser and detector settings using Scanware TCS version 5.1 software. Confocal images were visualized in section view mode. Integration analysis of images was employed with Scion Image Release Beta 3b (Scion Corporation, Frederick, MD, USA). The fluorescence intensity (mean density) averaged over each cell image is an indicator for the cellular uptake efficiency, i.e., higher density value means higher cellular uptake [25]. Data were averaged of three measurements for each cell image after subtracting the background (the image captured without the laser excitation) reading.

Results and discussion

The cellular uptake of liposomes is generally believed to be mediated by adsorption of liposomes onto the cell surface and subsequent endocytosis. Fusion of the liposomes with endosomal membranes appears to be necessary for the effective delivery of drug (gene) from the liposome to the cell. While it is still not known what factors define the uptake of liposomes by different cells, the different extents of binding for a given liposome composition by different types of cells suggest that the binding itself may be the crucial step [18].

CLSM

It is a technique for obtaining high-resolution optical images. The key feature of confocal microscopy is its ability to produce in-focus images of thick specimens, a process known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically-complex objects. CLSM gives us sharper images than those from conventional fluorescence microscopy techniques. Confocal microscopy also provides a substantial improvement in lateral resolution and the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation. CLSM offers a dramatic instrumental advantage for fluorescence microscopy through discrimination against out-of-focus background fluorescence, through inherent resolution perpendicular to the plane of focus and improved in-plane resolution [27]. Quantitative measurements of fluorescence intensity in such images can provide precise image determination of fluorescence marker distributions in three dimensions.

The cellular uptake of fluorescent-labeled liposomes by two cell lines is visualized by CLSM. The major advantage of CLSM is that the cell (tissue) can be optically sectioned, and that the distribution of the fluorescent probe in the cell can be visualized by images parallel to the surface of the cell. The section view mode is confirming cellular uptake and distribution of the fluorescent-labeled liposomes, since they will appear in all sections examined by CLSM. Without section view mode examination, the liposomes may be adsorbed on the cell surface and we cannot confirm their cellular uptake. The intra- and extracellular distributions of AVE-liposomes were observed by CLSM using appropriate excitation and emission filters and three fluorescence probes like Rh-PE, DiI and CF.

The extent of liposome uptake by cells was in general higher in the absence of serum [14]. We used cell medium with an addition of FCS 30% and 100% in some of these uptake experiments contrary to what is normally used of 5% or 10% FCS, in most of other previous works [11,15,22]. It is clear from Fig. 1D that the presence of 100% FCS, as

a cell culture medium, effectively reduces the liposomal uptake with Mewo cells.

FCS of 100% may cause liposome disruption as indicated in the background of Fig. 1C, where we noticed some disrupted parts of the labeled liposomes. In addition, we found that 100% FCS (instead of DMEM) may cause the Mewo cells to be rounded or flattened (not healthy) as the normal cells in DMEM medium. The mean densities were (224 ± 15.2), (82.44 ± 10.95), and (63.33 ± 7.18) for DMEM medium, DMEM medium supplemented with 30% FCS and 100% FCS, respectively. These data agree with that mentioned before [2,14]. Most mammalian cells require the addition of serum to the culture medium for optimal growth and maintenance of the cell lines in vitro.

As a result, serum components may be adsorbed to the cell surface or may be ingested by the cells during culturing, thus affecting cellular properties. It is quite conceivable, therefore, that interaction of liposomes with cultured cells is influenced by the presence of serum components on the cell surface or in the incubation medium.

The cellular uptake of AVE-liposomes with HepG2 cells was greater than that of Mewo cells (Fig. 2). The mean densities were (78.7 ± 24.26) for HepG2 cells and (43.3 ± 42.4) for Mewo cells. These data verify the dependence of liposomal uptake on the type of cells. Mady et al. [17] showed that at least two steps were involved in gene transfection mediated by AVE-liposomes. One was endocytosis, where the liposomes were internalized into target cells, and the other was membrane fusion between the liposome vectors and endosomes, where DNA is transferred from the liposome to the nucleus.

The results show that the AVE-labeled liposomes can be internalized into the cytoplasm of Mewo cells (Fig. 3). However, the liposomal lipid markers (Rh-PE and DiI) are accumulated more than aqueous content marker (CF), indicating selective accumulation of liposomal lipid with respect to its contents. This difference can be due to the possibility that some fraction of encapsulated CF may leak out from the liposomes during their interaction with cells. However, any CF leakage from the liposomes prior to endocytosis would cause apparently low values of liposomal endocytosis. The results show that the AVE-liposomes can deliver encapsulated fluorescent molecules (CF) and fluorescent lipid markers (Rh-PE, DiI) labeled liposomes into the cellular cytoplasm.

The fact that the examined liposomes were transferred into the cytoplasm, in the presence of serum, implies that disruption of the intracellular membranes or fusion

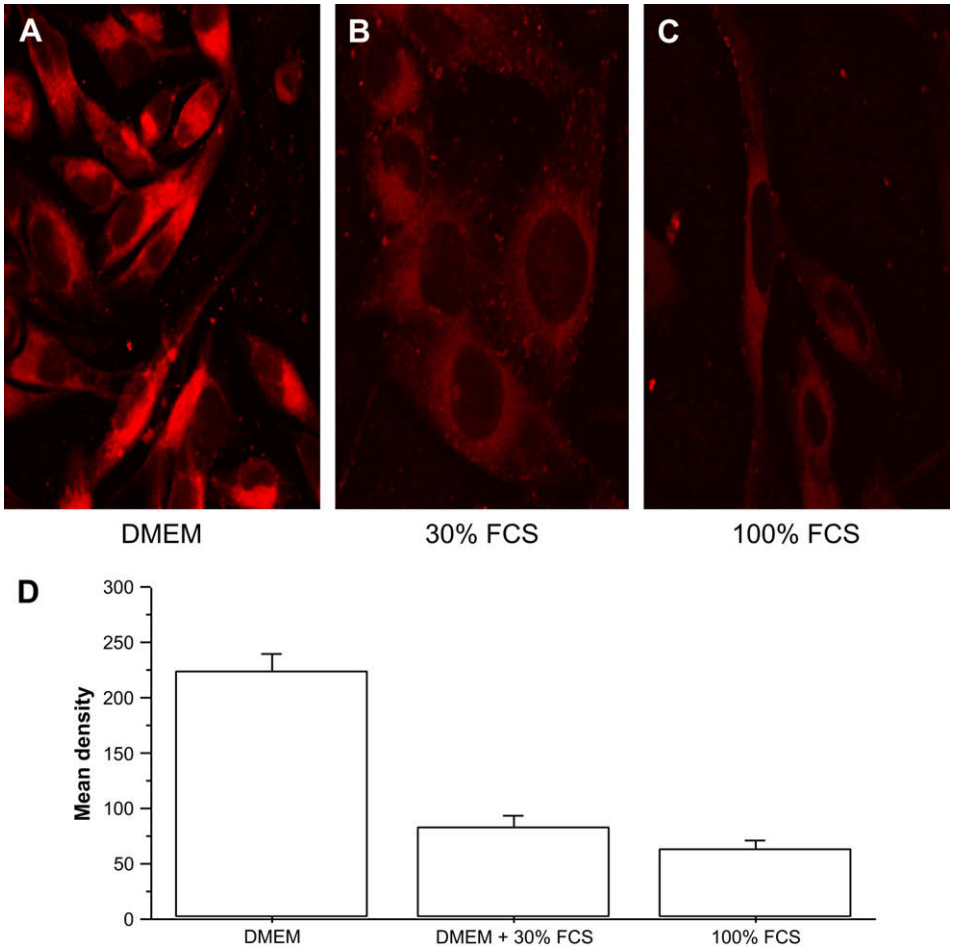

Figure 1 Confocal laser scanning microscopy of Mewo cells incubated with AVE-3 (Rh-PE) liposomes in DMEM supplemented with different %FCS (A—C), at 37 °C and 5% CO2 after 1 h. (D) Effect of FCS concentration (in cell culture medium) on the AVE-3 uptake by Mewo cells after 1 h incubation time.

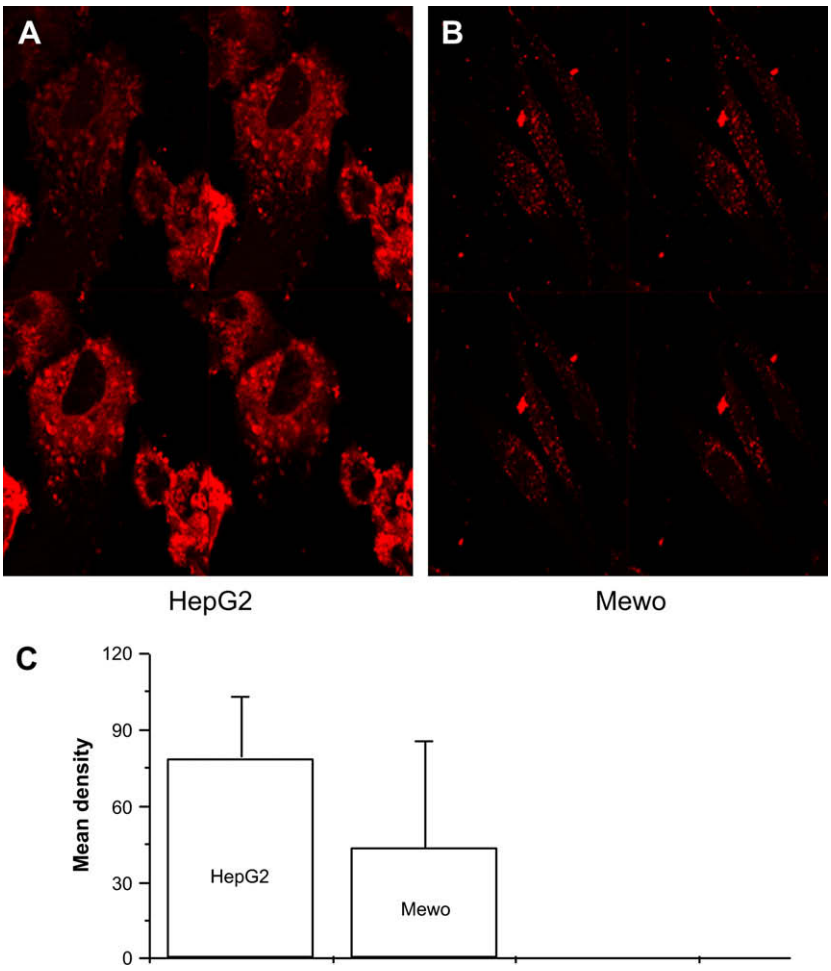

Figure 2 Confocal laser scanning microscopy of (A) HepG2 and (B) Mewo cells incubated with AVE-3 (DiI) liposomes in DMEM, at 37 °C and 5% CO2 after 2 h. (C) Dependence of liposomal uptake extent on cell type.

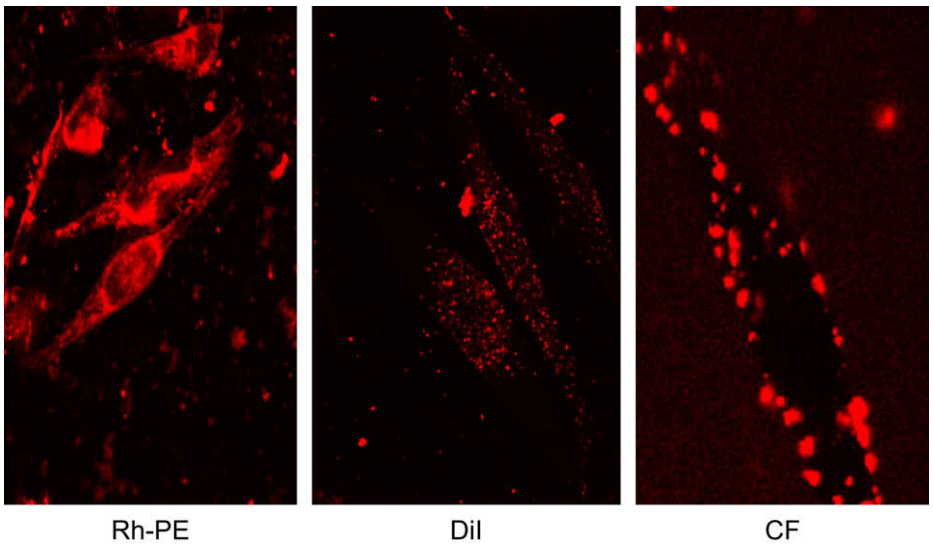

Figure 3 Confocal laser scanning microscopy of Mewo cells incubated with AVE-3 (different probes) liposomes in DMEM, at 37 °C and 5% CO2 after 2 h.

between the liposomal membrane and the intracellular compartment membrane is the most likely mechanism involved in the cytoplasmic delivery [17].

The basic conclusion from the work presented here, is that all examined cell lines, by using CLSM, are capable of internalizing the AVE-liposomes in the presence of serum and that the entrapped contents are released directly into the cellular cytoplasm. We conclude that there is a high efficiency of cytoplasmic delivery by AVE-liposomes to cells in culture. However, we believe that a better understanding of the AVE-liposome-cell interactions in vitro based on the information presented in this work will provide us with important clues to the behaviour of liposomes in vivo.

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