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Efficient Gene Delivery with Serum into Human Cancer Cells Using Targeted Anionic Liposomes

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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. Previous studies have shown an efficient, systemic trans-gene expression in many cell lines (in vitro) by using an anionic liposomal vector, based on the composition of retroviral envelopes (artificial viral envelopes, AVEs). The AVE-liposomes and their complexes with plasmid (DNA) were characterized according to zeta potential measurements and transmission electron microscopy (TEM). We successfully demonstrated that AVE liposomes, dispersed in 10% serum-containing growth medium, efficiently delivered plasmid DNA to HuH-7 (human hepatoma cell line) cells. We assessed the utility of liver-targeted vesicles as a drug/gene delivery system for the treatment of liver diseases. We found that small unilamellar AVE vesicles containing 15 mol% digalactosyl diglyceride (DGDG) are efficiently targeted to the liver via the hepatic asialoglycoprotein receptor.

INTRODUCTION

Gene therapy is based on the introduction of specific exogenous sequences of DNA into the target cells for production of the therapeutic gene product (Crystal, 1995; Lasic and Templeton, 1996). The prerequisite for successful gene therapy is efficient and safe delivery of DNA into the cells. Because of the fast progress of nucleic acid-based technologies in the treatment of diseases, the call for appropriate delivery vehicles becomes increasingly important. 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 (Shepushkin et al., 1997). The most widely used types of vehicles for gene delivery are: viral (e.g. adenovirus, retrovirus and adeno-associated virus) and non-viral (e.g. liposomes, polymer, peptides) (Lasic, 1999). 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 (Müller et al. 2001). Nonviral vectors, on the other hand, frequently face the problem of low transduction efficiency.

Liposomes and lipid-based drug delivery systems have been used extensively over the last decade to improve the pharmacological and therapeutic activity of a wide variety of drugs. More recently, this class of carrier systems has been used for the delivery of relatively large DNA and RNA-based drugs, including plasmids, antisense oligo-nucleotides and ribozymes (Semple et al., 1998). 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 (Felgner et al., 1994). 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 (Wang and Huang, 1987). Polylysine-condensed DNA entrapped into folate-targeted anionic liposomes have been successfully used for tumor cellspecific gene transfer (Lee and Huang, 1996).

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We introduced an attractive alternative in gene therapy by using an anionic artificial virus-like envelope (AVE) liposome for the encapsulation of polyethylenimine (PEI)-condensed DNA. AVEs mimic the lipid composition of retroviruses (Chander and Schreier, 1992). These natural lipids are anionic and, in contrast to their artificial cationic counterparts, interact only weakly with their biological environment and therefore are nontoxic (Müller et al., 2001). The efficiency and safety of the in vitro use of AVE-liposomes have been demonstrated in many cell lines (Gagne et al., 1998; Welz et al., 2000; Müller et al., 2001; Nahde et al., 2001; Fahr et al., 2002). These AVE-liposomes have special fusogenic properties, allowing them to transport encapsulated or associated drugs into cells. Recently, nuclear localization of oligonucleotides 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 (Gagne et al., 1998; Welz et al., 2000). Müller et al. (2001) 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.

One of the most attractive sites for gene transfer is the liver because it plays a major role in many metabolic processes and is involved in a large variety of diseases. Improving gene transfer efficiency may be critical for ex vivo hepatocytes gene therapy, especially for autologous hepatocyte transplantation. When hepatocytes are procured from donors and cultured ex vivo, a certain fraction of hepatocytes do not survive. For ex vivo hepatocytes gene therapy, liposomes are good vectors because of their availability and lack of limitation in transfection gene size. Indeed, Sorgi et al. (1997) demonstrated that protamine sulfate enhances liposome-mediated gene transfer into several types of cells in vitro, but not specifically in hepatocytes. So, in this study, we tried to investigate the transfection in hepatocytes by using different targetors.

Most mammalian cells require the addition of serum to the culture medium for optimal growth and maintenance of the cell lines in vitro. The presence of serum often reduces the transfection efficiency of liposomal vectors (Felgner et al., 1987; Felgner and Ringold, 1989; Gao and Huang, 1995; Lee and Huang, 1997). This may be due to the premature release of DNA from the complexes or its degradation by the nucleases. So, in the presence of serum, we investigated that AVE liposomes efficiently delivered DNA to HuH-7 cells.

In order to improve upon these therapies in the future, clinically active liposome delivery systems most likely will need to include site-directed surface ligands to further enhance their selective delivery. In an attempt to develop gene delivery system acceptable to all hepatocytes-associated disorders, the transfection efficiency with sugar-grafted AVE liposomes has been studied in vitro. We could quantitate the transfection efficiency (using FACS analysis) of AVE-liposomes conjugated with different sugar moieties and complexed with PEI/DNA for targeting these complexes to the hepatocytes asialoglycoprotein receptor.

MATERIALS AND METHODS

Materials

1, 2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE) and dioleoyl phosphatidyl serine (DOPS) were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Low-molecular-weight, branched PEI is obtained from Lupasol G100, BASF, Ludwigshafen, (Germany). Cholesterol (CHOL) was obtained from Calbiochem (La jolla, CA, USA). Cholesteryl-beta-D-glucoside (gal. Chol) is synthesized by P. Holig (Institute of Pharmaceutical Technology and Biopharmacy, Philipps University, Marburg, Germany); monosialoganglioside-GM1 (from Bovine Brain) and digalactosyl diglyceride (DGDG) (from whole wheat flour) were purchased from Sigma, Mo, USA. D-Galactosyl-β1-1′-N-octanoyl-D-erythrosphingosine (C8 β-D-galactosyl ceramide) is obtained from Avanti Polar Lipids Inc. (USA).

Cell Culture

Fetal calf serum (FCS), L-Glutamine (200 nM solution), Penicillin 5000 units/streptomycin 5000 μg/ml, and Dulbecco's modified Eagle's medium (DMEM) were obtained from Bio Whittaker Europe, Verviers, Belgium. HuH-7 cells were obtained from Children Hospital, Friedrich-Alexander-Universiy, Erlangen, Germany. PEGFP-C1 plasmid is purchased from Clontech Laboratories, Inc., Hiedelberg, Germany. H2B-GFP plasmid from Dr Teru Kanda, La Jolla/USA; while DNA analogues of chimeric Propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR USA). All other reagents were of analytical reagent grade.

Preparation of Liposomes

AVE liposomes are composed of equimolar amounts of DLPE, DOPS and CHOL (Welz et al., 2000). Liposomes were formulated according to well-established methods of extrusion (Olson et al., 1979). 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 lipid suspension was extruded through 100 nm polycarbonate membranes (Nucleopore GmbH, Germany), using a commercially available extruder (Liposofast, Avestin Inc., Canada). Size measurement was done by dynamic laser light scattering (Zetasizer 3000 HS, Malvern Instruments, Germany) and the size was in the range of 100 nm. AVE-liposomes included different targeted sugars (15 mol% DGDG, 5 mol% ceramide,

5 mol% GM1, 5 mol% gal-chol) for targeting to the hepatocytes asialoglycoprotein receptor.

Electron Microscopy

AVE liposomes and their complexes with PEI/DNA were also characterized by using negative stain electron microscope (EM 109, Zeiss, West Germany). On a copper grid, we added the appropriate concentration from each sample. Then add one drop of 20% uranyl acetate; wait for 2 min at room temperature; remove the excess solution with a filter paper; then examine under the electron microscope.

Zeta Potential

In deionized water, we dispersed the pure AVE liposomes and their complexes with PEI/DNA (20.7:1 $+/-$) and then measured the corresponding zeta-potential ζ by using Zetasizer 3000 HS, Malvern Instruments, Germany. The liposome size was $\cong 100$ nm. The lipid concentration was 10 μg lipid/2.5 μg DNA.

DNA Transfection Experiment

To investigate the gene expression or transfer efficiency, the cells were seeded at a density of (1×10^5) on 6-well plates. HuH-7 cells were grown in DMEM medium supplemented with 10% FCS, 1% glutamine and 1% penicillin–streptomycin solutions. The transfection system and cells were incubated for 6 h at 37°C in 5% CO_2. The cells were washed away by rinsing thrice with cold PBS. Cells were fixed with formaldehyde solution (3.7% in PBS) and stained with PI (Molecular Probes, USA). The GFP-expressing cells were visualized under the fluorescent microscope (Leica DM R with A4, L5, N3 and Y5 filters, Leica, Wetzlar, Germany) with spot fluorescence image systems digital camera (Intas, Göttingen, Germany) after 6 h transfection time. Integration analysis of images was employed with Scion Image Release Beta 3b (Scion Corporation, Frederick, MD, USA). The mean density measurement is an indicator for the transfection efficiency (uptake), i.e. high mean density value means higher efficiency (uptake) (Welz et al., 2000). We evaluated the transfection efficiency of the AVE-liposomes with respect to (i) transfection efficiency (ii) contribution of the ligand to HuH-7 cells transfection and (iii) cell type specificity. For this purpose, a plasmid carrying a pEGFP-C1 promotor-driven nuclear green fluorescent protein (GFP) gene was condensed with PEI 5000 and packed into AVE liposomes.

FACS Analysis of GFP Expression

HuH-7 cells were grown on 10-cm dishes and transfected for 6 h with AVE/PEI/DNA complexes and AVE conjugated with different targeted sugars (5 mol% GM1; 5 mol% ceramide; 5 mol% gal-chol and 15 mol% DGDG).

The cells were trypsinized 18 h later, washed once with PBS, fixed in ice-cold 75% ethanol for 1.5–2 h on ice incubation, resuspended in PBS, treated with RNase A (Roche, Mannheim, Germany) for 1 h in room temperature, and stained with propidium iodide (20 μg/ml) for at least 10 min. The cells were analyzed by flow cytometry (DAKO Galaxy, Hamburg, Germany) using a laser excitation at 488 nm and emission at 516 nm (for propidium iodide) and at excitation of 488 nm and emission of 568 nm (for GFP).

RESULTS AND DISCUSSION

Transmission Electron Microscopy (TEM)

Negative stain electron microscopy is a useful method for addressing questions concerning size distribution of liposome, and although obtaining quantitative data is laborious, negative staining is a reliable technique, which is simple to perform and requires only limited specialized equipment, which should be available in any electron microscopy laboratory. In addition, negative staining can also provide information on whether liposomes produced in a particular manner are multi- or unilamellar (Haschmeyer and Myers, 1972).

Electron micrographs of AVE-liposomes and their complexes with PEI/DNA (20.7:1 $+/-$) were shown in Fig. 1. The majority of the particles in the micrographs appeared spherical and small (≤ 150 nm in diameter). The PEI/DNA complex showed a mixture of oblong, smaller things and larger round structures, while the PEI/DNA/AVE complex shape ranged from elongated rod-shaped to ball-shaped particles. DNA can be condensed into a toroidal structure (20–30 nm) upon neutralization of approximately 90% of its charge (i.e. its phosphate residues) (Wilson and Bloomfield, 1979). One possible mechanism by which the complex may have formed is illustrated in Lee and Huang (1996). In this model, DNA is first condensed into a cationically charged complex with PEI. The cationic complex is then entrapped into anionic liposomes by spontaneous charge interaction.

Zeta Potential Measurements

Transfection complex formation is based on the interaction of the positively charged polycations with the negatively charged phosphate groups of the nucleic acid. The size and surface charge density of transfection complexes can be related to the transfection efficiency of a reagent. The information of the overall charge of transfection complexes by zeta-potential measurements can be speed up by the development of better non-viral DNA delivery vectors for gene therapy (Son et al., 2000). Values of the zeta-potential of liposomes indirectly reflect vesicle surface net charge and can therefore be used to evaluate the extent of interaction of the liposomal surface anionic charges with the pre-condensed DNA. On this

AVE

PEI/DNA (20.7:1 +/-)
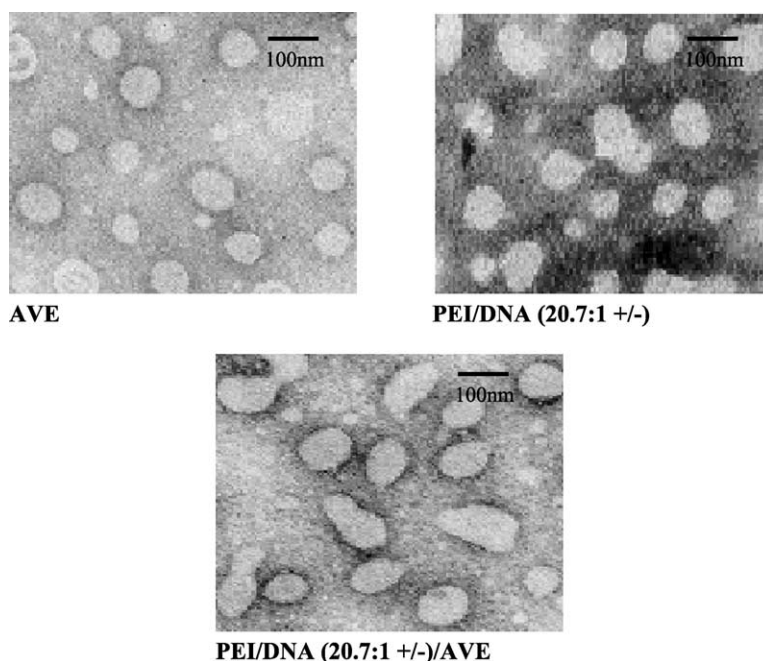
PEI/DNA (20.7:1 +/-)/AVE

FIGURE 1   Negative stain electron micrographs of AVE liposomes and their complexes with PEI/DNA.

basis, the zeta-potential of AVE liposomes was investigated before and after complexing with PEI/DNA complexes.

Results in Fig. 2 show the negatively zeta-potential of the naked plasmid (-23.5 ± 3.7 mV) and for AVE liposomes was also negative (-8.16 ± 1.7 mV), n = 5. After adding PEI to DNA plasmid in charge ratio of ($20.7:1 +/-$), the zeta-potential value becomes positive (14.4 ± 0.9 mV). It becomes negative after adding liposomes to PEI/DNA complex and has a value of (-2.5 ± 0.1 mV). The following physical, chemical parameters were used in the determination of zeta-potential: medium viscosity 0.89 cP, medium refractive index 1.333, temperature 25°C and dielectric constant 79.

With most cationic gene delivery systems, a net positive charge promotes the unspecific interaction of the cationic complex with cell surface and plasma proteins (Roerdink et al., 1983). Negatively charged liposomes also interact with the biological environment in a nonspecific manner, but a liposomal gene delivery system with a net negative surface potential should exhibit less nonspecific tissue uptake and a better overall biocompatibility than cationic carrier systems (Roerdink et al., 1983; Müller et al., 2001).

DNA Transfection

Little is known about the durability of plasmid DNA transgene expression in mammalian cells in the absence
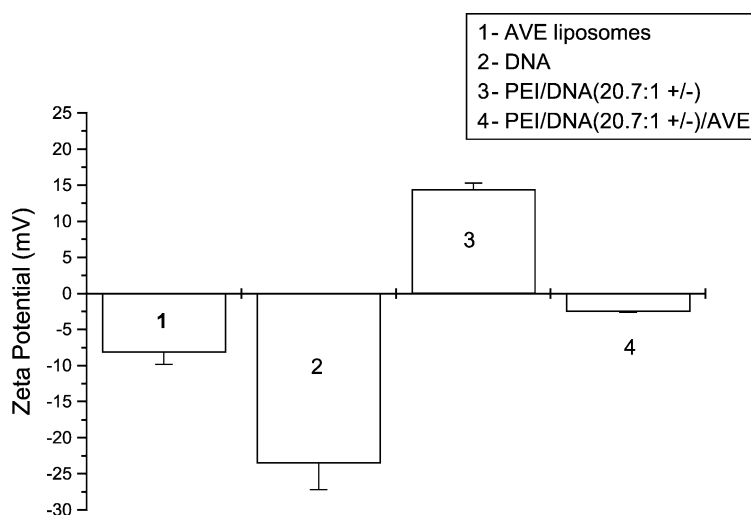

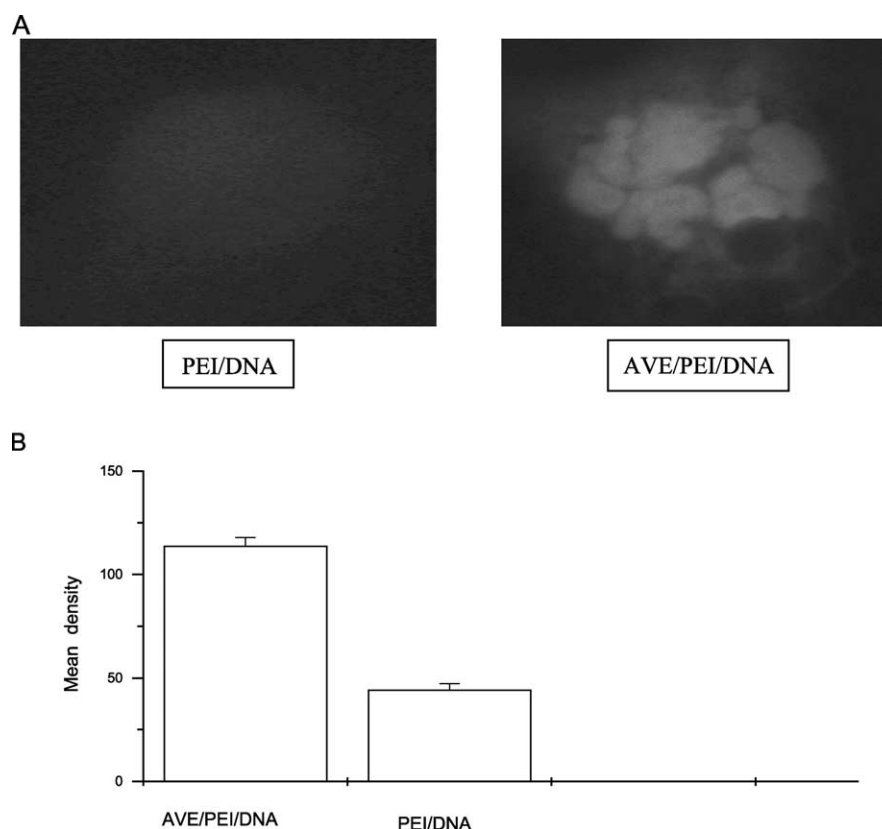FIGURE 2   Zeta potential measurements of different formulations, (n = 5).

FIGURE 3   (A) Transfection of HuH-7 cells with pEGFP plasmid. Cells were exposed for 6 h to different complexes in DMEM medium supplemented with 10% FCS at 37°C and 5% CO_2 and fluorescence microscopy was performed after 24 h in the culture medium. (B) AVE increases the transfection efficiency of PEI/DNA (20.7:1 $+/-$) complexes.

of growth selection. For this purpose, we have begun the study of the liposomal transfer of plasmid DNA encoding GFP in HuH-7 cells. GFP expression was noticeable in cells within 6 h of transfection. GFP expression is adequate for the identification, isolation and monitoring of stable transfection events after lipid-mediated transfection of eukaryotic cells. Fluorescence microscopy and FACS analysis revealed the molecular mechanism of gene transfection by AVE-liposomes. The results showed that at least two steps were involved in gene transfection mediated by AVE-liposomes. One was endocytosis, where the liposome–DNA complex was internalized into target cells, and the other was membrane fusion between the liposome vectors and endosomes, where DNA transferred from the liposome to the nucleus.

Fig. 3A,B showed that HuH-7 cells were successfully transfected by AVE/PEI/DNA complex and expressed GFP. The mean densities (arbitrary units) of the green color in the nucleus were (113.83 ± 4.1) and (44.33 ± 2.9) for cells transfected with AVE/PEI/DNA and PEI/DNA complexes, respectively. There was no or only a minor detectable fluorescence in the nucleus in the case of the control experiment with naked DNA. These experiments were performed in complete cell culture medium containing 10% FCS, indicating that the transfection using AVE-liposomes was not inhibited by serum proteins and AVE-liposomes, dispersed in 10% serum-containing

growth medium, efficiently delivered a plasmid DNA into HuH-7 cells.

The ability of PEI to transfect a wide variety of cells is well established. Boussif and his co-workers have documented PEI-mediated transfection in 25 different cell types, including 18 human cell lines as well as pig and rat primary cells (Boussif et al., 1996).

The ratio of PEI nitrogens to DNA phosphates is important in terms of transfection efficiency and cell toxicity. At charge ratio of 20.7 $(+/-)$ PEI/DNA, which was chosen for plasmid transfection experiment, DNA condensation with PEI was completed. Also, no indication for cell toxicity could be detected at this charge ratio as investigated recently (Müller et al., 2001).

FACS Analysis of GFP Expression

One approach to improve liposomes as gene delivery system is to increase their specific distribution in target tissues and organs by including site-directed surface ligands. This information can be used to optimize vesicle-mediated drug delivery to the liver. The asialoglycoprotein receptor-positive liver parenchymal cells selectively take up galactosylated AVE-liposomes in vitro. Pre-condensed DNA-galactosylated AVE-liposome complexes show higher DNA transfection into the liver parenchymal cells than DNA complexes with bare AVE-liposomes (Fig. 5).
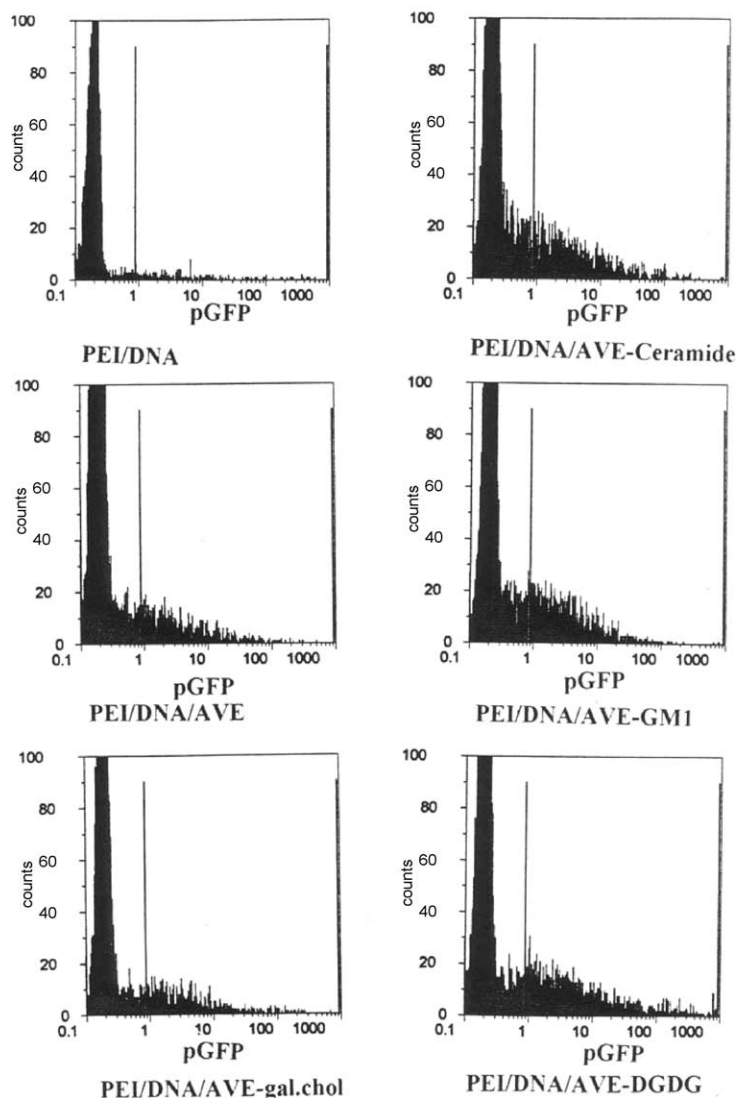
FIGURE 4 FACS analysis of HuH-7 cells, 24 h after transduction with AVEs (± sugars ligands) carrying a CMV-H2BGFP plasmid. Cells (in DMEM supplemented with 10% FCS) were exposed for 6 h, at 37°C and 5% CO_2, to the complexes and FACS analysis was performed after another 18 h in normal medium. PEI/DNA (20.7:1 +/−).

To quantitate the results of fluorescence microscopy study, GFP was monitored by FACS analysis and the results recorded as means of the main GFP peak. Fluorescence, expressed in arbitrary units, was recorded as the ratio of the fluorescent signal produced by transfected cells compared with untransfected cells (Fig. 4). The data in Fig. 5 clearly confirm the high transfection efficiency already seen in Fig. 4. The results clearly show that depending on the target motif on the surface of AVE liposomes, the entry of genes into liver cells is greatly enhanced. A more efficient GFP expression with the complexes containing AVE-DGDG, as much as 25% of the cell population, was observed. Dragsten et al. (1987) investigated that small unilamellar vesicles containing 15-mol% DGDG are efficiently targeted to the liver via the hepatic asialoglycoprotein receptor. AVE liposomes targeted with DGDG thus represent an efficient targeted gene therapy system for delivering encapsulated genes specifically to the liver. Jarnagin et al. (1992) showed that the transfection efficiency in hepatocytes was 10% by using DOTMA/DNA complexes. In contrast, GFP expression was seen in only 8.46% of cells transfected with PEI/DNA complexes and 13.63% of cells transfected with AVE (lacking the sugar) containing complexes, and these levels of expression were considerably lower than that seen with ligand-carrying AVEs.

We also investigated that gal-chol, GM1 and galactose ceramide (containing AVEs) increase the transfection efficiency to 14.19, 20.33 and 21.455%, respectively. Although these results clearly indicate a role for the targeted sugars in the transfection of HuH-7 cells, the significant extent of transfection seen with, for example, AVE-DGDG points to the greater accessibility of the terminal galactosyl moiety of the DGDG.

The specific composition of the targeted AVEs, in particular, the use of physiologic, anionic lipids, suggests
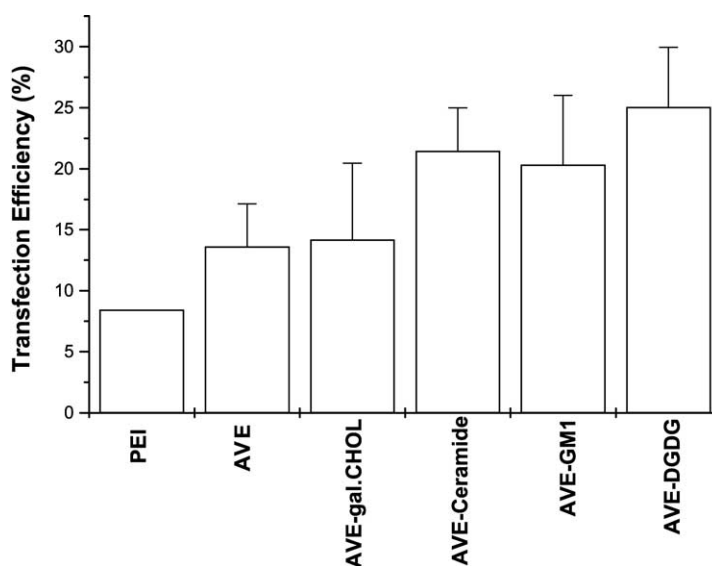
FIGURE 5   Transfection of HuH-7 cells with AVEs (conjugated with targeted sugars) carrying a CMV promotor H2BGFP plasmid: summary of FACS analysis determining the fraction of GFP-positive cells. For details, see Fig. 4.

that the vector system should also be applicable in vivo and might have some advantages over previously tested liposomal vectors. We, therefore, believe that these targeted liposomal vectors represent a true advance in the field of vector development. The benefits of this system will be maximal in liver diseases, such as liver cancer and hepatitis, for which available drugs exhibit significant extra-hepatic toxicity, low hepatic clearance rates, and/or are rapidly metabolized before reaching their site of action.

Acknowledgements

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