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Characterization of Human Serum Albumin-Facilitated Lipofection Gene Delivery Strategy

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Abstract

We report the characterization of a facilitated lipofection strategy, which employs a formulation prepared with human serum albumin, DMRIE-C and pCMVβ. The transfection complexes in the lipofection formulation containing albumin were characterized for size by light scattering, intracellular trafficking by confocal microscopy, and uptake mechanism by inhibitors. Supplementation of the formulation of DMRIE-C plus pCMVβ with albumin enhances lipofection efficiency 8-9 fold and the size of the complexes 2-2.5 fold as measured by light scattering. Analysis of intracellular trafficking of the transfection complexes by confocal microscopy reveals colocalization of DNA and albumin in the cytoplasm and the nucleus. Pretreatment of the cells with chlorpromazine or excess albumin, cytochalasin B or filipin complexes results in inhibition of the transfection efficiency by 20%, 55%, or none, respectively. The results suggest a moderate involvement of clathrin, a significant involvement of actin-associated macropinocytosis, and no involvement of caveolae in the uptake of the transfection complexes prepared with human serum albumin, DMRIE-C and pCMVβ.

Keywords: Facilitated lipofection; Human serum albumin; Cationic liposome; Endocytosis; Macropinocytosis; Clathrin

Introduction

The liposome-based gene transfer strategy is one of the most studied nonviral gene delivery strategies [1]. While cationic liposomes do not show the adverse effects exhibited by viral vectors such as host immune response and insertional mutagenesis, they suffer from low transfection efficiency [2]. To improve the lipofection efficiency, it is imperative to understand the gene transfer mechanism. To date, there is only limited understanding of the cellular and molecular mechanisms of cationic lipid-mediated gene transfer [3]. This communication reports characterization of the mechanism of an albumin-facilitated lipofection gene delivery strategy.

Our lab was the first to demonstrate that supplementation of liposome with transferrin greatly enhanced lipofection efficiency [4]. Subsequently, we showed that other ligands, such as insulin, epidermal growth factor and lectins [5,6], also enhanced lipofection efficiency. This phenomenon was confirmed by several other labs working with transferrin *in vivo* [7,8] and *in vitro* [9-12], fusigenic peptides [9-11], human serum albumin [13], integrin-binding peptide [14,15], and anti-transferrin receptor antibody [16,17]. Employing the transferrin-facilitated lipofection strategy to deliver p53 gene intratumorally in a human prostate cancer xenograft, suppression of tumor growth and increased animal survival were observed [18]. Similar observations were made in p53 gene therapy of a head and neck tumor xenograft model [8] and a breast cancer metastasis model [16] by intravenous delivery of a liposome-based formulation containing transferrin, and head and neck and prostate cancer xenograft model using a lipofection formulation containing anti-transferrin receptor antibody [17]. These results indicate that liposome supplemented with a protein ligand or a receptor antibody is a promising nonviral vector for cancer gene therapy.

Cationic liposome-mediated gene delivery was originally thought to occur by fusion of the positively charged liposome in the complex with the plasma membrane. Subsequently, endocytosis was suggested as the main cell internalization pathway of DNA-cationic lipid complexes [3]. To date, very little is known about the endocytosis mechanism for the facilitated lipofection. The enhancement of the lipofection efficiency by a ligand was thought to result from increased binding of the ligand in the transfection complexes to its specific receptor [4,7,8]. However, failure of an excess amount of free human serum albumin (HSA) to significantly inhibit the transfection efficiency of HSA-lipoplexes suggested that the uptake of the HSA- lipoplexes through binding of albumin to its putative receptor was not the major gene delivery mechanism for the HSA-facilitated lipofection [13]. Therefore, alternative mechanisms might be involved in the uptake of the ligand-liposome-DNA complexes.

In this study, we optimize the albumin-facilitated lipofection formulation and elucidate its uptake mechanism utilizing the endocytosis mechanism-specific chemical inhibitors, including chlorpromazine, excess HSA, filipin complexes, and cytochalasin B. Our results indicate a moderate involvement of the clathrin, no involvement of caveolae, and a significant involvement of intact filamentous actin in the internalization of the albumin-DC-DNA transfection complexes.

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Material and Methods

Materials

DMRIE-Cholesterol (DC) was purchased from Invitrogen (Carlsbad, CA). Human serum albumin and filipin complexes were purchased from Sigma (St. Louis, MO). Cytochalasin B and chlorpromazine were purchased from MP Biomedicals (Aurora, OH). pCMVβ plasmid DNA was purchased from BD Biosciences Clontech (Mountain View, CA). *Label* IT CX-Rhodamine Nucleic Acid Labeling Kit was purchased from Mirus (Madison, WI). Fluorescein Isothiocyanate for labeling of HSA was purchased from Pierce (Rockford, IL).

Cell culture

A human pancreatic cancer cell line, Panc 1, was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Panc 1 cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) (all Gibco, Gaithersburg, MD) at 37°C in a humidified 5% CO_2 atmosphere.

Agarose gel electrophoresis analysis of transfection formulation

For optimization of DC to DNA ratio, 1 µg of pCMVβ plasmid was incubated with varying amounts of DC for at least 15 min at RT prior to loading the samples onto a 1% agarose gel. To optimize the ratio of HSA to DC-DNA complexes, varying amounts of HSA were mixed with 5.5 µg DC, followed by incubation for 15 min at room temperature (RT). After the addition of 1 µg DNA, the formulations were incubated at least 15 min prior to loading onto a 1% agarose gel. For DNase treatment of the transfection complexes, 2 µl (0.1 u/µl) of RQ1 RNase-Free DNase (Promega, Madison, WI) was added to the formulations prepared as described above, followed by incubation for 15 min at RT prior to analysis on 1% agarose gel.

Transfection

Panc 1 cells were grown to approximately 80% confluency in a 48-well culture plate. The standard transfection formulation was prepared as described previously (4). Briefly, in 12 X 75- mm polystyrene tubes (Becton Dickson, Lincoln Park, NJ), the following reagents were added sequentially, gently mixed and incubated for 15 min at RT after each addition: 100 µl HBS containing 0.05 µg HSA and 5.5 µg DC; and 100 µl HBS containing 1 µg of the 7.2 kb pCMVβ plasmid DNA. This formulation was designated HSA plus DC plus DNA. The DC plus DNA formulation was prepared by mixing 100 µl HBS containing 5.5 µg DC and 100 µl of HBS containing 1 µg of pCMVβ. The 48-well plate was washed once with 1 ml HBS. The mixture was transferred to each well, which had been covered with 0.3 ml of serum-free MEM without antibiotics. After the plate had been incubated for a predetermined length of time, the transfection solution was replaced with 1 ml MEM containing 10% FBS and antibiotics. Approximately 48 h later, cells were washed once with HBS, the protein was harvested with M- PER®. Mammalian Protein Extraction Reagent (Pierce), and measured for β-galactosidase (β-gal) activity spectrophotometrically using All-in-One Mammalian β-galactosidase Assay Reagent (Pierce) on a UVmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Protein was measured by the Bradford method with Coomassie® Plus Protein Assay Reagent (Pierce) using bovine serum albumin (HSA) as the standard. Data were expressed as nanograms β-galactosidase per microgram protein.

Light scattering measurements

The size of the complexes in various formulations was measured by light scattering using the ZetaPlusAnalyzer (Brookhaven Instruments Corporation, Holtsville, NY). These measurements were made at RT on five formulations, DC; DC plus DNA; DC plus 0.05 µg HSA; 0.05 µg HSA plus DC plus DNA (DNA added last); and 10 µg HSA plus DC plus DNA.

Confocal microscopy

Panc 1 cells cultured on 18-mm glass coverslips placed in a 12-well plate were transfected with 1 ml of the standard formulation using 1 µg of FITC-HSA, 5.5 µg DC, and 1 µg of Rhodamine-DNA. Rhodamine-DNA was prepared with the Mirus Label IT-Rhodamine Labeling Kit following the manufacturer's directions and FITC-HSA was prepared with FITC from Pierce following the manufacturer's described method. Six hours after transfection, the transfection solution was removed and complete MEM was added. After six hours of incubation, the medium was removed. Then, the coverslips were washed three times with PBS, fixed in 4% paraformaldehyde at room temperature for 10 min, and rinsed three times with PBS and two times with sterile water. The coverslips were placed face down on a glass slide over 6 µl of Vectashield (Vector Laboratory, Inc., Burlington, CA) and their edges were sealed with nail polish. The slides were examined under a Zeiss LSM 410 confocal laser scanning microscope (Carl Zeiss, Inc, Goettinger, Germany) equipped with an argon/krypton laser. Z-sectioning was performed to identify the section that included the nucleus. A composite picture of the differential interference contrast (DIC), Rhodamine and FITC images was constructed.

Endocytosis inhibitor treatment protocol

Endocytosis inhibitors were prepared to a working stock concentration 50 times that of the final concentration. Prior to the standard transfection protocol, the media were removed from each well and the cells were washed once with 1 ml HBS. Following the removal of HBS, 10 µl of the stock solution of the endocytosis inhibitor was added to 0.5 ml of pretreatment solution (0.2 ml HBS and 0.3 ml medium without antibiotics) and added to each well. After 30 min, the pretreatment solution was replaced with 0.5 ml of the transfection solution containing 0.2 ml of the transfection formulation and 0.3 ml of serum-free MEM without antibiotics. Subsequent procedure follows that described under "**Transfection**."

Fluorescent microscopy

Panc 1 cells were cultured on 18-mm glass coverslips placed in a 12-well plate. After mounting as described above, the slides were viewed on a Zeiss Axiovert 200 M microscope equipped with an ORCA-ER (Hamamatsu) digital camera and processed with Slidebook 4 Digital Microscopy software (Intelligent Imaging Innovations, Inc., Denver, CO).

For the transferrin uptake experiment, Panc 1 cells were washed two times with PBS, and serum starved for 30 min at 37°C in the absence or presence of chlorpromazine or pretreatment solution containing 0.5% BSA. Next, 10 µg/ml Texas Red-transferrin (Molecular Probes) was added to each well, and incubated for 5 min at 37°C. Following washing of the coverslips three times with PBS, cells were fixed with 4% paraformaldehyde at RT for 10 min. Then, coverslips were washed three times with PBS, two times with water, and then mounted face down onto glass slides with 6 µl Vectashield (Vector Laboratories, Inc.) and sealed with nail polish.

For examination of cholera toxin subunit B uptake into Panc 1 cells, cells were washed two times with HBS, and incubated for 30 min at 37°C with pretreatment solution containing 0.1% BSA with no additives or 5 µg/ml filipin complexes. Cells were cooled to 15°C and incubated in the same medium containing 2.5 µg/ml Alexa Fluor 488-Cholera Toxin subunit B (Molecular Probes) for 30 min. Cells were then incubated at 37°C for 30 min. After washing three times with PBS and fixing with 4% paraformaldehyde at RT for 10 min, coverslips were then washed three times with PBS and two times with water and mounted onto glass slides as described above.

To stain actin, Panc 1 cells were washed two times with HBS, and pretreated 30 min at 37°C in the absence or presence of cytochalasin B in pretreatment solution. After the pretreatment solution was removed and cells were washed two times with PBS, they were fixed with 4% paraformaldehyde at RT for 10 min. After washing three times with PBS, the cells were permeablized by treatment with 0.1% Triton X-100 in PBS containing 1% BSA at RT for 4 min, and then washed two times with PBS. The cells were then treated with 400 µl of the Alexa Fluor 488-phalloidin (Molecular Probes) solution prepared as described next and incubated at room temperature for 1 h. To 1 ml PBS containing 1% BSA, 25 µl of methanolic stock of Alexa Fluor 488-phalloidin was added, which was followed by vortexing and centrifugation. Coverslips were then washed five times with PBS and two times with water and mounted as described above.

Results

Optimization of albumin-facilitated lipofection formulation

To determine the optimal ratio of the DMRIE-Cholesterol (DC)

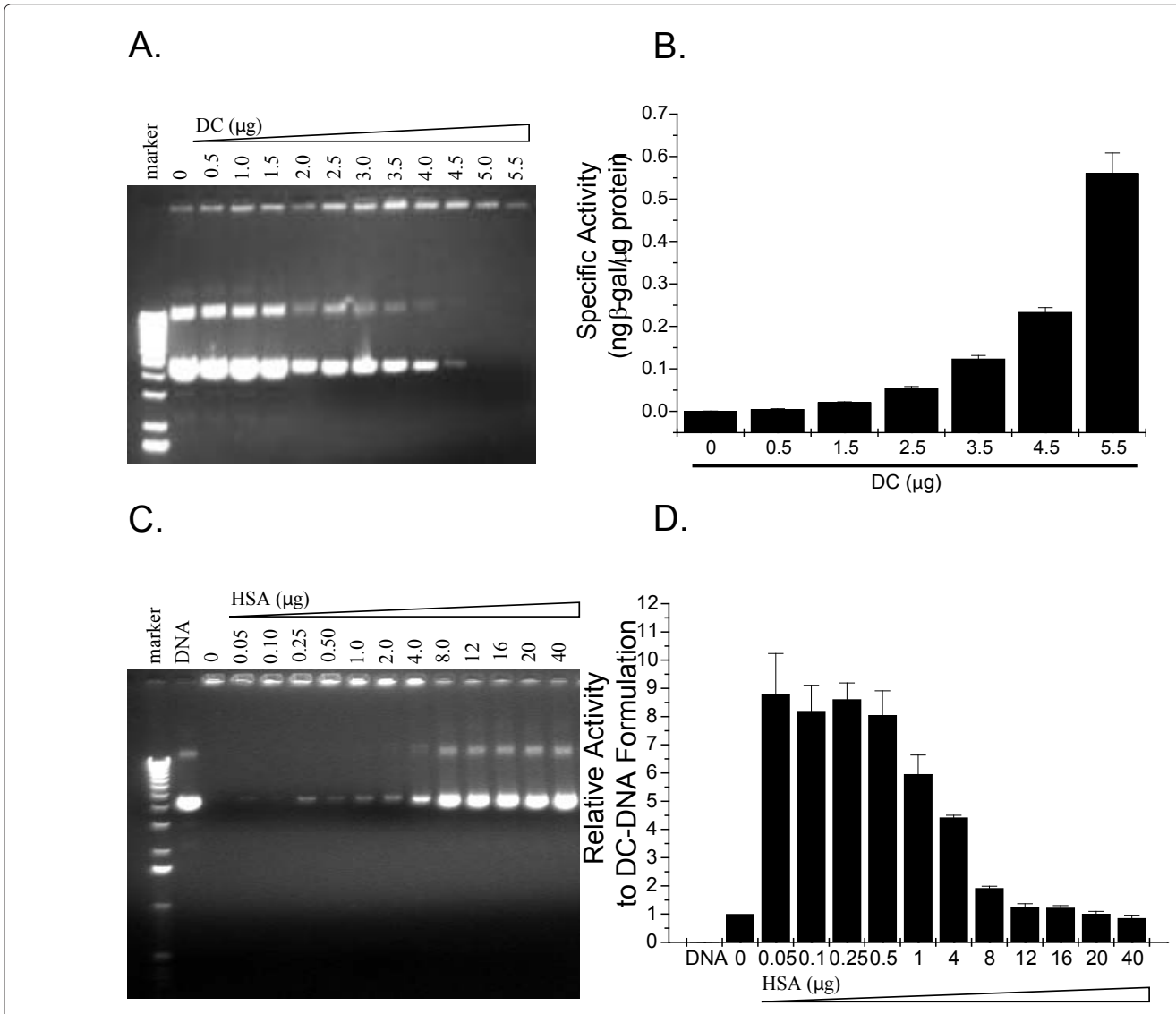

Figure 1: Optimization of albumin-facilitated lipofection formulation. (A) Agarose gel electrophoresis assessment the complex formation in the formulations containing varying amounts of DC at a fixed amount of pCMVβ (1 µg). (B) Panc 1 cells were transfected for 5 h with formulations containing 1 µg DNA plus the various amounts of DC indicated and assayed for β-galactosidase activity in the cells after cultured for 48 h. (C) Agarose gel electrophoresis assessment of the complex formation in the formulations containing various amounts of HSA with 5.5 µg DC and 1 µg DNA. (D) Panc 1 cells were transfected for 5 h with formulations containing 5.5 µg DC, 1 µg DNA plus different amounts of HSA from 0.05 to 40 µg. The data are expressed as nanogram β-galactosidase per microgram of total cell protein (mean ± standard error (SE) obtained from triplicate wells).

liposome with pCMVβ DNA, DC at various amounts was mixed with 1 μg of pCMVβ followed by analysis by agarose gel electrophoresis and transient transfection assay. As shown in Figure 1A, 5.0-5.5 μg DC fully conjugated 1 μg pCMVβ. The transient transfection assay showed that the transfection efficiency increased in a DC concentration-dependent manner. The formulation containing 5.5 μg DC plus 1.0 μg pCMVβ yielded the highest transfection efficiency (Figure 1B). Although the formulation containing 6.5 μg DC plus 1.0 μg pCMVβ yielded higher transfection efficiency than the formulation with 5.5 μg DC plus 1.0 μg pCMVβ, it exhibited toxicity. Therefore, the formulation containing 5.5 μg DC and 1 μg pCMVβ was used for optimization of the HSA-facilitated lipofection formulation.

To determine the optimal dose of HSA for the HSA-facilitated lipofection formulation, BSA at varying amounts was sequentially mixed with 5.5 μg DC and 1 μg pCMVβ, followed by analysis of the complexes by agarose gel electrophoresis and assay for transient transfection efficiency in Panc 1 cells. The majority of the plasmid DNA remained fully conjugated when up to 4 μg of HSA was used (Figure 1C). When the amount of HSA reached 8 μg and above, most of the DNA was found not to be associated with the complexes. Transfection of Panc 1 cells with these formulations showed the highest transfection efficiency with formulations containing 0.05-0.5 μg HSA (Figure 1D). The transfection efficiency of the optimal formulation containing HSA in this range was 8-9 times that of the DC plus DNA formulation. The transfection efficiency of the formulation containing HSA at ≥ 12 μg was down to the level of DC plus DNA formulation. The formulation prepared with 0.05 μg HSA, 5.5 μg DC, and 1.0 μg DNA was used for subsequent studies. It was noted that although the degree of enhancement of lipofection efficiency by albumin varied with different batches of liposome and with respect to the measured specific activity, the standard HSA-facilitated lipofection formulation remained more efficient than the formulation without albumin.

Resistance of DNA in the lipofection complexes to DNase degradation

Liposome with or without transferrin was known to protect the DNA in the liposome (± transferrin)-DNA complexes from DNase digestion [19]. We wanted to know if the DNA in the HSA-DC-DNA complexes was susceptible to DNase digestion. As shown in Figure 2, DNA in either the DC plus DNA or HSA plus DC plus DNA formulation was resistant to DNase degradation while free DNA was completely degraded.

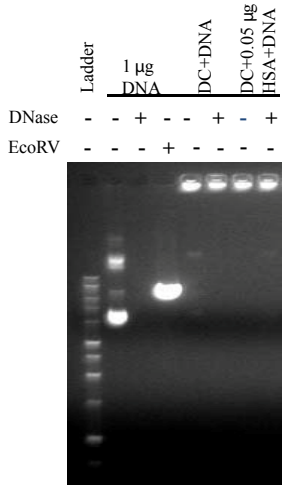

Figure 2: Protection of pCMVβ in the albumin-facilitated lipofection formulation from DNase degradation. DNA, DC plus DNA, DC plus HSA plus DNA formulations were treated with and without 0.2 u of RQ1 RNase-Free DNase at RT for 15 min and then analyzed by electrophoresis on a 1% agarose gel. EcoRV treatment linearized the plasmid DNA.

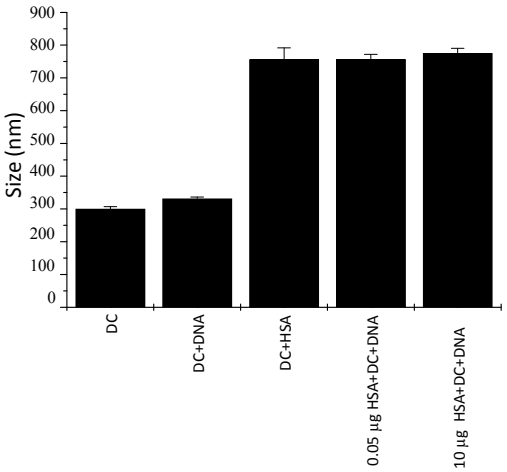

Figure 3: Particle size measurements of various transfection formulations. The formulations including DC; DC plus DNA; DC plus 0.05 μg HSA; 0.05 μg HSA plus DC plus DNA (DNA added last); and 10 μg HSA plus DC plus DNA (DNA added last) were measured for size by light scattering. Results are expressed as mean diameter ± standard error (SE) (n=3).23.

Mean particle sizes of the transfection complexes

The mean particle sizes of liposome alone, liposome with either HSA or plasmid DNA, and liposome with HSA and plasmid DNA in solution were measured by dynamic light scattering. The DC alone formulation had a diameter of 300.1 ± 7 nm (Figure 3). pCMVβ plasmid DNA plus DC had a diameter of 331.8 ± 4.4 nm. DC plus 0.05 μg HSA had a diameter of 756.2 ± 35.5 nm. The 0.05 μg HSA plus DC plus DNA formulation had a diameter of 756.9 ± 15.2 nm. The transfection complexes formulated with 10 μg HSA plus DC plus DNA had a diameter of 775.6 ± 14.8 nm.

Colocalization of HSA and DNA in the nucleus by confocal microscopy

Confocal microscopy was employed to assess the intracellular trafficking of the transfection complexes in Panc 1 cells prepared with FITC-labeled HSA, DC, and Rhodamine (Rh)-labeled pCMVβ. The cells were treated for 6 h with this formulation followed by incubation in the routine culture medium for an additional 6 h before serial Z-sectioning. Overlays of both fluorophores revealed colocalization of HSA and DNA not only in the cytoplasm and perinuclear space but also in the nucleus (Figure 4A and 4B).

Mechanisms for the uptake of the transfection complexes in Panc 1 cells

The three major endocytosis pathways that have been identified in non-phagocytic mammalian cells employ clathrin [20], caveolae [21], and macropinocytosis [22]. These pathways are known to be inhibited by chlorpromazine [23,24], sterol-binding agents, such as filipin complexes [25], and actin polymerization-inhibition agents, such as cytochalasin B [26-28], respectively. These inhibitors were

employed to characterize the uptake mechanisms of the transfection complexes containing 0.05 µg HSA plus 5.5 µg DC plus 1 µg DNA. As shown in Figure 5A, Panc 1 cells pretreated with chlorpromazine prior to transfection resulted in an approximately 21% decrease in transfection efficiency. Pretreatment of these cells with excess amounts of HSA, which assessed whether an albumin receptor was involved in the uptake of the transfection complexes, also caused a 20% decrease in transfection efficiency. Treatment of Panc 1 cells with filipin complexes had no effect on transfection efficiency, while treatment with cytochalasin B caused a 55% decrease in transfection efficiency.

To demonstrate that these inhibitors worked in Panc 1 cells and the experimental results were valid, morphological changes of these cells were monitored by fluorescence microscopy following treatment

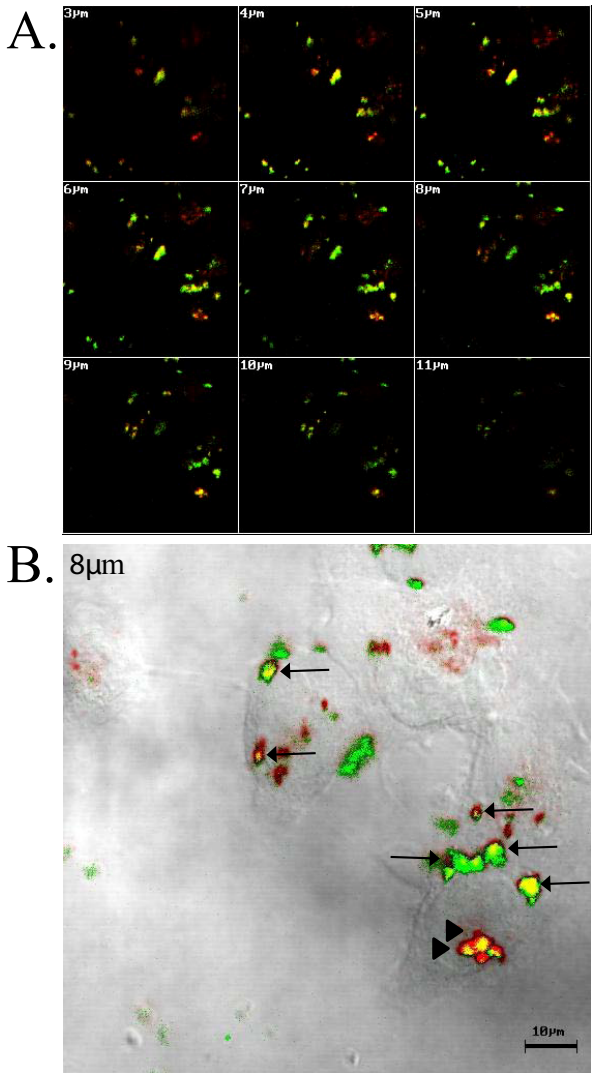

Figure 4: Localization of transfection complexes in transfected Panc 1 cells by confocal microscopy. Panc 1 cells were transfected with the standard formulation prepared with FITC-HSA plus DC plus CX-Rhodamine (Rh)-DNA, then incubated for an additional 6 h with complete culture medium. (A) FITC and Rh overlays of the 3- to 11-µm sections of 1 µm serial Z-sections starting from the bottom of the cells. (B) Composite image of the differential interference contrast, FITC, and Rh at the 8-µm Z-section. The arrowheads indicate colocalization of HSA(FITC) and DNA(Rh) inside the nucleus and the arrows indicate the complexes in the cytoplasm and/or perinuclear space.

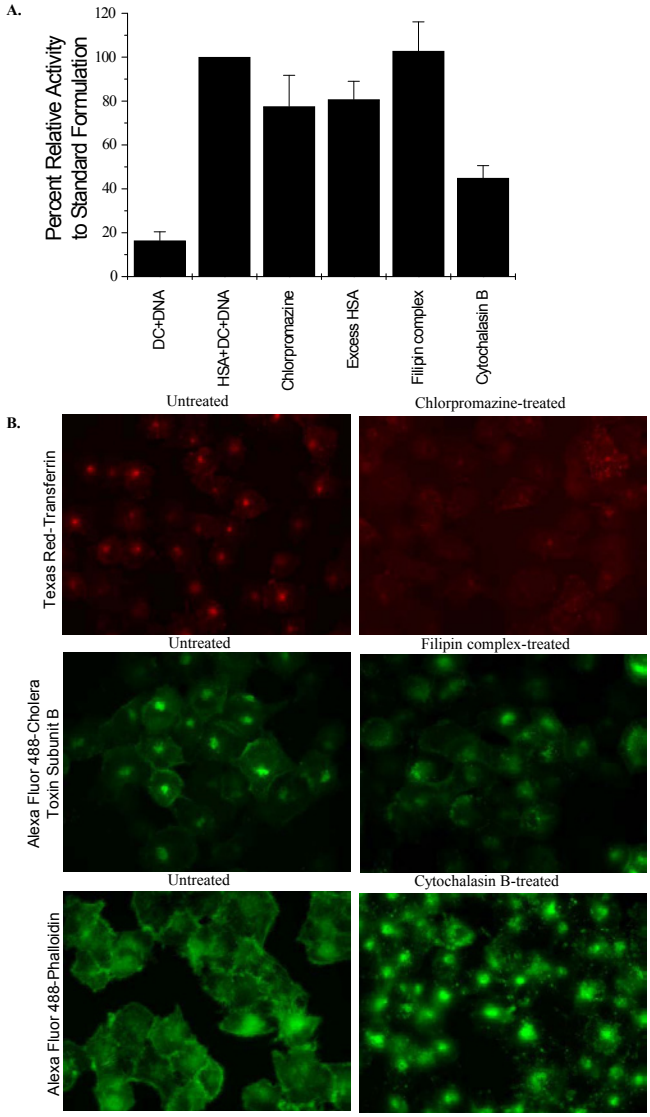

Figure 5: Effect of endocytosis inhibitors on transfection (A) Panc 1 cells were pretreated with chlorpromazine (100 µM), excess HSA (225 µg), filipin complexes (5 µg/ml), or cytochalasin B (125 µM) for 30 min prior to transfection with the standard formulation. The transfection efficiency of the cells transfected with the standard formulation was set as 100%. Mean ± S.E. were obtained from three separate experiments carried out in triplicate. Effect of endocytosis inhibitors on uptake of fluorescein-labeled material. (B) Fluorescence micrographs of Panc 1 cells treated without and with chlorpromazine prior to treatment with 10 µg/ml Texas Red-conjugated transferrin; filipin complexes prior to treatment with 2.5 µg/ml Alexa Fluor 488-cholera toxin subunit B; and cytochalasin B prior to treatment with 1 unit of Alexa Fluor 488-Phalloidin to identify F-actin.

with these inhibitors as well as fluorescein-labeled markers. As shown in the top panel of Figure 5B, untreated Panc 1 cells internalized the clathrin-mediated endocytosis marker, Texas Red-transferrin, and chlorpromazine treatment greatly decreased its uptake. The effect of filipin complexes on caveolae-mediated endocytosis was examined by the internalization of fluorescein-labeled cholera toxin subunit B. While most of the untreated cells appear to internalize cholera toxin B subunit (Figure 5B, middle panel), the number of cells which took up cholera toxin and the intensity of intracellular fluorescence were decreased in

filipin complexes-treated cells. As shown in the bottom panel of Figure 5B, pretreatment of the cells with cytochalasin B resulted in a complete disruption of the actin cytoskeleton as shown by the loss of the radiated actin stress fibers in the cytoplasm displayed in the untreated cells.

Discussion

Low transfection efficiency is a major limitation of non-viral gene delivery strategy. To overcome this limitation, it is essential to understand its gene transfer mechanism. In this communication, we report the optimization of a human serum albumin-facilitated lipofection formulation, the characterization of the transfection complexes, and elucidation of mechanisms of uptake of the transfection complexes in Panc 1 cells. Our results show that supplementation of 5.5 μg DC plus 1.0 μg pCMVβ formulation with 0.05 to 0.5 μg HSA yields the highest transfection efficiency in Panc 1 cells. This result is at variance with a previous report in which 32 μg of HSA was found to be the optimal amount for enhancing the lipofection efficiency of the DOTAP:DOPE/DNA (1:1) formulation in COS-7 cells [13]. Differences in cell lines and cationic liposomes used in these two studies may account for such a difference. These results suggest that maximal lipofection efficiency achieved in a cell type may be liposome-specific for a given ligand, indicating a need for optimization of the facilitated lipofection formulation for each cell type.

Similar to our previous observation that supplementation of lipofectin with transferrin increased the sizes of the transfection complexes [20], the HSA plus DC plus DNA formulation contains complexes with sizes 2-2.5 times those of DC alone or DC-DNA complexes as measured by dynamic light scattering (Figure 3). Since the mean size of the transfection complexes in all lipofection formulations that contain HSA is the same regardless of the amounts of HSA used (Figure 3), the size of the complexes is determined by the liposome and HSA. Further, since the transfection efficiency is influenced by the amounts of HSA (Figure 1D), the size of the transfection complexes alone is not the primary determinant of the transfection efficiency of the complexes. It is of interest to note that increasing the amount of HSA above 4 μg prevents the DNA from participating in the formation of the "active" transfection complexes (Figure 1C). Although competition of the binding of HSA with DNA for the liposome is an explanation, a detailed mechanism remains to be elucidated.

Similar to the results obtained previously by our lab for the transferrin-facilitated lipofection [20], human serum albumin is found to co-localize with DNA in the nucleus as well as the cytoplasm and perinuclear space by confocal microscopy. This result suggests that HSA and DNA are transported together to the nucleus. However, the mechanism for the nuclear transport of HSA-DNA complexes remains to be elucidated.

We are also surprised to observe that while chlorpromazine treatment greatly inhibits the uptake of Texas Red-labeled transferrin (Figure 5B), only a moderate decrease (i.e. about 21%) of the transfection efficiency is found in chlorpromazine-treated cells. This observation suggests that clathrin-mediated endocytosis is only moderately involved in the cellular uptake of the HSA-DC-DNA complexes. In A549 pneumocytes and HeLa cervical carcinoma cells, internalization of FITC-poly-L-Lysine-labeled DOTAP/DNA lipoplexes was inhibited by chlorpromazine, and transfection by the lipoplexes was completely abolished by inhibiting clathrin-mediated endocytosis [29]. These results suggest that clathrin-mediated uptake of the lipoplexes is the major mechanism of endocytosis for these complexes in A549 and HeLa cells, but not a major mechanism in Panc 1 cells. The difference may be due to differences in cell lines and lipofection formulations utilized in these studies.

When an excess amount of HSA is used to treat the cells prior to transfection with the albumin-facilitated lipofection formulation, only moderate inhibition (20%) of the transfection efficiency is observed. The degree of inhibition is similar to that (16%) reported in COS-7 cells [13]. These results suggest the presence of a protein on the plasma membrane to which albumin binds and facilitates approximately 16-20% of the cellular uptake of the albumin-liposome-DNA complexes in these two cell lines. It will be of interest to identify such a receptor for albumin. A membrane protein such as gp60 (or albondin) present in endothelial cells is a potential candidate. It should be pointed out that the degree of inhibition by excess albumin is consistent with the result of the chlorpromazine treatment in Panc 1 cells.

The decreased uptake of fluorescein-conjugated cholera toxin subunit B in Panc 1 cells treated with filipin complexes indicates that this uptake mechanism is fully functional in these cells. However, failure of filipin complexes to inhibit lipofection efficiency indicates that caveolae are not involved in the endocytosis of the lipofection complexes. A similar observation was reported for the internalization of FITC-poly-L-lysine-labeled DOTAP/DNA lipoplexes in A549 and HeLa cells [29].

Cytochalasin B pretreatment results in a dramatic disruption of actin filaments and causes a large decrease in transfection efficiency, suggesting that actin plays a major role in the uptake of the transfection complexes. A similar observation was made in COS-7 cells [13]. One possible explanation for the inhibition observed after cytochalasin B pretreatment is through inhibition of macropinocytosis. Macropinocytosis has been observed in a variety of metazoan cells, operating in macrophages and many tumor cells constitutively, and in other cells after stimulation with growth factors or phorbol esters [27]. It has been previously suggested that actin may spatially organize endocytic hotspots, perhaps serving as an anchor for scaffold assembly. Alternatively, actin assembly might serve to propel nascent endocytic vesicles through the dense cytoskeletal apparatus that lines the cell cortex [30].

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