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Human serum albumin enhances DNA transfection by lipoplexes and confers resistance to inhibition by serum

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Received 2 June 1999; received in revised form 4 October 1999; accepted 24 November 1999

Abstract

Cationic liposome–DNA complexes (‘lipoplexes’) are used as gene delivery vehicles and may overcome some of the limitations of viral vectors for gene therapy applications. The interaction of highly positively charged lipoplexes with biological macromolecules in blood and tissues is one of the drawbacks of this system. We examined whether coating cationic liposomes with human serum albumin (HSA) could generate complexes that maintained transfection activity. The association of HSA with liposomes composed of 1,2-dioleoyl-3-(trimethylammonium) propane and dioleoylphosphatidylethanolamine, and subsequent complexation with the plasmid pCMVluc greatly increased luciferase expression in epithelial and lymphocytic cell lines above that obtained with plain lipoplexes. The percentage of cells transfected also increased by an order of magnitude. The zeta potential of the ternary complexes was lower than that of the lipoplexes. Transfection activity by HSA-lipoplexes was not inhibited by up to 30% serum. The combined use of HSA and a pH-sensitive peptide resulted in significant gene expression in human primary macrophages. HSA-lipoplexes mediated significantly higher gene expression than plain lipoplexes or naked DNA in the lungs and spleen of mice. Our results indicate that negatively charged HSA-lipoplexes can facilitate efficient transfection of cultured cells, and that they may overcome some of the problems associated with the use of highly positively charged complexes for gene delivery in vivo. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Gene therapy; Cationic liposome; Human serum albumin; Macrophage; In vivo; Gene delivery

1. Introduction

The efficient delivery of functional therapeutic genes into target cells in vitro and in vivo is an im-

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plications [3–7]. Relatively stable expression has been achieved in several tissues with this system [8–12]. Among the advantages of lipoplexes are lack of immunogenicity, safety, ability to package large DNA molecules, and ease of preparation [4,5]. Their disadvantages include limited efficiency of delivery and gene expression, toxicity at higher concentrations, potentially adverse interactions with negatively charged macromolecules in serum and on cell surfaces, and impaired ability to reach tissues beyond the vasculature unless directly injected into the tissue [4,13]. Lipoplexes may be coated in vivo with serum proteins, such as lipoproteins or immunoglobulins, or bind non-specifically to cells such as erythrocytes, lymphocytes and endothelial cells, as well as to extracellular matrix proteins [3,4,13]. This will limit the ability of the complexes to reach target tissues and cells.

We considered the possibility that coating cationic liposomes with the most abundant plasma protein, albumin, might alleviate some of the undesired interactions between liposome–DNA complexes and serum components. In support of this hypothesis, a recent study on the effects of serum components on cationic liposome–oligonucleotide complexes found that bovine serum albumin can prevent the dissociation of the complexes induced by certain polyanions [14]. Previous observations by Cheng [15] and our laboratories [16,17] had indicated that transferrin complexed to lipoplexes enhances gene delivery to various cell types, including primary cells. Studies on the mechanisms of gene delivery by such transferrin–lipoplexes suggested, however, that specific ligand–receptor interactions are not involved in this process [6,18]. Thus, although albumin would not be expected to function as a receptor ligand [19], it could still facilitate transfection by lipoplexes, possibly by mediating endocytosis similar to that by transferrin. Earlier reports had also described the ability of albumin to promote membrane fusion under acidic conditions [20]. Therefore, besides its potential role in triggering internalization of the lipoplexes, albumin may also function as a fusogenic protein that destabilizes endosomes under acidic conditions, thus enhancing intracellular gene delivery.

2. Materials and methods

2.1. Cells

HeLa and COS-7 cells were maintained at 37°C, under 5% CO₂, in Dulbecco’s modified Eagle’s medium-high glucose (DME-HG) (Irvine Scientific, Santa Ana, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO), penicillin (100 U/ml), streptomycin (100 µg/ml) and l-glutamine (4 mM). For transfection, 0.2 × 10⁵ HeLa cells, or 0.4 × 10⁵ COS-7 cells, were seeded in 1 ml of medium in 48-well culture plates and used at either 80–90% (HeLa) or 40–60% (COS-7) confluence. For transfection 0.3 × 10⁵ cells were seeded in 1 ml of medium in 48-well culture plates and used at 40–60% confluence. H9 cells, a CD4⁺ clonal derivative of the Hut-78 T-cell line readily infectable by HIV [21], were grown in RPMI 1640 medium (Irvine Scientific) supplemented with 10% (v/v) FBS, l-glutamine (2 mM), and antibiotics as above. B-lymphocytic TF228.1.16 cells that stably express functional HIV envelope proteins on the cell surface [22] were grown in DME-HG supplemented with 16% (v/v) FBS, l-glutamine (4 mM) and antibiotics. Human peripheral blood monocyte-derived macrophages were prepared as described previously [23]. In some experiments, human granulocyte-macrophage colony stimulating factor (hGM-CSF) (Boehringer Mannheim Biochemica, Indianapolis, IN) was added to the wells (final concentration of 100 IU/well) on the second day following isolation. In other experiments, cells were cultured for 8 more days in medium containing 20% FBS, antibiotics and l-glutamine, but in the absence of hGM-CSF.

2.2. Cationic liposome–DNA complexes

Unilamellar cationic liposomes were prepared from a 1:1 mixture (by weight) of 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL), by extrusion of multilamellar liposomes through polycarbonate filters of 50 nm pore diameter, and filter-sterilization (Millex 0.22 µm filters), as described [16]. Complexes were prepared by sequentially mixing 100 µl of a solution of 100 mM NaCl, 20 mM HEPES, pH 7.4, with or
without human serum albumin (HSA, Sigma) with liposomes (2.1, 4.2, or 8.4 μg of total lipid, depending on the +/- charge ratio) and incubated at room temperature for 15 min. One hundred microliters of buffer containing 1 μg of pCMVluc (VR-1216; a gift of Dr. P. Felgner (Vical, San Diego, CA)) or 1 μg pCMV-SPORT-β-gal plasmid (Gibco-BRL Life Technologies, Gaithersburg, MD) were then added and gently mixed; the mixture was further incubated for 15 min at room temperature. Quaternary complexes containing both HSA and the fusogenic peptide GALA (a 30 amino acid, pH-sensitive, amphipathic peptide with the sequence WEAALAEALA-EALAEHLAEALAA) [24,25] were prepared by adding the protein and the peptide sequentially to the liposomes, followed by the initial 15-min incubation, and the addition of the plasmid. The zeta potential of the various complexes was measured in a Coulter DELSA 440 instrument as described previously [16].

2.3. Transfection of cells

Transfection activity was measured as described previously [16]. Lipid/DNA complexes were incubated for 4 h with the cells in serum-free medium, unless indicated otherwise. The medium was then replaced with the appropriate medium containing FBS, as described in the figure legends, and the cells were further incubated for 48 h. The level of gene expression in cell lysates (obtained with lysis buffer, Promega, Madison, WI) was evaluated by measuring luciferase activity using a scintillation counter protocol (Promega) and a standard curve for luciferase activity. The protein content of the lysates was measured by the DC Protein Assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. The data were expressed as ng of luciferase per mg of total cell protein. Transfection efficiency was evaluated by scoring the percentage of cells expressing β-galactosidase [16]. Cell viability following transfection under the different experimental conditions was quantified by a modified Alamar Blue (AccuMed, Westlake, OH) assay [16,26].

2.4. Transfection in vivo

Gene expression in vivo was assessed by injecting HSA-lipoplexes, plain lipoplexes (both containing DOTAP/cholesterol at a 1:1 mol ratio [11]) or naked DNA into 8-week-old mice (Charles River) via the tail vein in a volume of 200 μl. Plain lipoplexes or HSA-lipoplexes (containing 3.2 mg HSA) were prepared at a charge ratio (+/-) of 2/1 and contained 100 μg pCMVluc. The lungs and spleen were harvested and homogenized 8 h following injection, and luciferase expression in the supernatant was measured in a luminometer (Mediators Diagnostika, Vienna, Austria; www.mediators-int.com) using a standard curve for luciferase activity.

3. Results

3.1. Enhancement of transfection activity and efficiency by human serum albumin (HSA)

We examined the effect of HSA on transfection mediated by cationic liposome–DNA complexes at different lipid/DNA (+/-) charge ratios. An enhancement of transfection was observed as the amount of albumin associated with (1/1) DOTAP:DOPE/DNA complexes was increased up to 32 μg (Fig. 1). Doubling this amount led to a decrease in the level of luciferase gene expression, indicating that 32 μg of HSA is optimal for transfection. Although increasing amounts of albumin still enhance transfection as compared to plain lipoplexes (controls) it also reduces the amount of DNA that can interact with the cationic liposomes, since it reduces the net surface charge on the liposomes. Thus, amounts of albumin above 32 μg appear to reduce the amount of DNA associated with the liposomes to a sufficient degree to counteract its enhancing effect. Further studies were therefore performed with 32 μg of HSA. In a separate series of experiments, the role of HSA in transfection by different lipid/DNA (+/-) charge ratios was examined (Fig. 2A). HSA enhanced transfection by all the lipid/DNA charge ratios tested. This enhancement was particularly pronounced for the net negatively charged (-/+) complexes, since the plain lipoplexes were essentially ineffective. The highest levels of transfection were obtained with the 1/1 (theoretically neutral) lipid/DNA charge ratio. This observation suggests that a net positively charged lipid/DNA complex is not re-
quired to obtain high levels of transfection, and that higher positive to negative charge ratios do not enhance gene transfer or expression mediated by HSA. A similar tendency was also observed for the plain lipid/DNA complexes. Zeta potential measurements revealed that the HSA-associated complexes are more negatively charged than the corresponding plain lipoplexes. For the 1/1 (+/−) charge ratio, the zeta potential was 1.4 ± 5.8 mV for the plain lipoplexes, and −25.6 ± 6.1 mV for the HSA-lipoplexes. For the 1/2 charge ratio the zeta potential of the HSA-lipoplexes was −33.8 ± 6.0 mV. These data indicate that HSA facilitates gene delivery by net negatively charged complexes.

The association of HSA with the lipoplexes also resulted in a significant enhancement of the levels of luciferase expression in HeLa cells, although to a lower extent than in COS-7 cells (data not shown). HSA-lipoplexes at the 1/1 (+/−) charge ratio were again the most effective of the different charge ratios, but the luciferase levels were around 170 ng/mg protein, compared to about 1400 ng/mg in the case of COS-7 cells. The association of HSA with the lipoplexes resulted in an increase of the number of cells that were detectably transfected. In the case of the 1/1 (+/−) charge ratio complexes, the percentage of cells expressing detectable β-galactosidase increased from 2% for plain lipoplexes to 20–25% for HSA-lipoplexes. For the 1/2 (+/−) complexes, the percentage increased from 0 to 5–10%, and for the 2/1 (+/−) complexes it increased from 1 to 5%, respectively. These results indicate that HSA-lipoplexes enhance both transfection activity (level of luciferase expression) and transfection efficiency (the percentage of cells transfected). It should be noted that our experiments were not designed to maximize the efficiency of transfection, but to explore the roles of HSA and the +/− charge ratio in transfection activity and efficiency.

3.2. Transfection of lymphocytes by HSA-lipoplexes

Adoptive cellular immunotherapy based on the use of genetically modified T-cells represents a promising strategy to increase the immune response against viral infections and malignant diseases, as well as to correct single gene defects in T-cell immunodeficiency syndromes [27–29]. HSA-lipoplexes greatly enhanced
gene expression in the T-lymphocyte cell lines H9 (Fig. 2B) and TF228.1.16 cells (data not shown), over that obtained with plain lipoplexes.

3.3. Transfection of primary macrophages by HSA-GALA-lipoplexes

Although considered as being cells that are very difficult to transfect by non-viral vectors, macrophages are crucial targets for gene therapeutic interventions since they are involved in a large variety of biological processes and pathologies [30-32]. We found that HSA-lipoplexes were considerably more effective than plain lipoplexes in transfecting macrophages (Fig. 3). We previously showed that the use of transferrin in combination with the pH-sensitive peptide GALA greatly enhanced the transfection of macrophages by lipoplexes, presumably by both pro-
motion of internalization of the complexes and disruption of the endosome membrane, respectively [17]. We therefore examined the effect of associating both albumin and GALA with cationic liposome-DNA complexes on transfection of macrophages derived from human blood monocytes. DOTAP:DOPE liposomes were mixed with 32 µg HSA or with its mixture with 0.6 µg of GALA, and then complexed with 1 µg of pCMVLuc plasmid. hGM-CSF (100 IU/ml) was added to the macrophages on the second day of differentiation. Other experimental details were as in Fig. 1, except that the cells were incubated for 48 h in medium containing 20% of FBS after the removal of the complexes.

Fig. 3. Effect of the association of HSA, in the presence or absence of the fusogenic peptide GALA, with cationic liposome-DNA complexes on transfection of macrophages derived from human blood monocytes. DOTAP:DOPE liposomes were mixed with 32 µg HSA or with its mixture with 0.6 µg of GALA, and then complexed with 1 µg of pCMVLuc plasmid. hGM-CSF (100 IU/ml) was added to the macrophages on the second day of differentiation. Other experimental details were as in Fig. 1, except that the cells were incubated for 48 h in medium containing 20% of FBS after the removal of the complexes.

3.4. Effect of serum on transfection

Transfection of certain cell types by some cationic liposome compositions is sensitive to the presence of serum [33,34]. The inhibition of gene delivery by serum is considered to be one of the limitations to the use of lipoplexes in vivo [34]. We therefore examined the effect of serum on the levels of transfection mediated by HSA-lipoplexes. The transfection activity mediated by the plain lipoplexes at the 2/1 (+/-) charge ratio was completely inhibited in the presence of serum (Fig. 4), while that mediated by HSA-lipoplexes was slightly enhanced. In the case of 1/1 (+/-) complexes, the presence of serum did not affect significantly the level of transfection by plain lipoplexes, but it enhanced transfection by HSA-lipoplexes.

3.5. Studies on the mechanisms of transfection mediated by HSA-lipoplexes

Previous studies have indicated that endocytosis is the major pathway of cellular entry of plain lipoplexes [35–37]. To gain insights into the internalization pathway followed by the HSA-lipoplexes, HeLa cells were pretreated before transfection with agents that interfere with various aspects of the endocytic pathway: (1) a mixture of antimycin A, NaF and NaN₃, which, by restricting the metabolic activity of the cell, strongly inhibits both receptor- and non-receptor-mediated endocytosis [38,39]; and (2) cytochalasin B, a drug that is known to disrupt the microfilament network by inhibiting actin polymerization, thereby blocking phagocytosis and pinocytosis, but not receptor-mediated endocytosis [40,41]. In addition, to evaluate whether the fusogenic proper-
ties of albumin also play a role in facilitating intracellular gene delivery from within endosomes, experiments were also carried out in the presence of bafilomycin A1. Being a specific inhibitor of the vacuolar ATPase proton pump, this drug prevents the acidification of the endocytic pathway [42, 43].

Although varying with the type of drugs used and the charge of the complexes tested, an inhibitory ef-

Fig. 5. Effect of different inhibitors on transfection by HSA-lipoplexes. HeLa cells were incubated for 30 min at 37°C, in the absence of serum, with a mixture of antimycin A (1 µg/ml), NaF (10 mM) and NaN₃ (0.1%), with cytochalasin B (25 µg/ml), or with bafilomycin A₁ (125 nM). COS-7 cells were incubated for 30 min at 37°C with an excess of free HSA (8 mg/0.3 ml). Cells were further incubated for 1 h at 37°C with HSA-lipoplexes in the presence of the various drugs or free HSA, and then washed once with serum-free medium. The medium was then replaced with DME-HG containing 10% FBS, and the cells were further incubated for 48 h before evaluation of transfection. The data are expressed as the percentage of luciferase activity in untreated controls for the two charge ratios tested (n.d., not determined).
fect on transfection was evident for essentially all the conditions (Fig. 5). The inhibitors of endocytosis strongly inhibited transfection by the HSA-lipoplexes at both charge ratios, the highest level of inhibition being observed with the 1/1 charge ratio. Although, in this study, these agents have been used to inhibit endocytosis, we cannot rule out that they may have other unknown effects on the transfection machinery. Cytochalasin B also had significant inhibitory effects on transfection. Inhibition of endosome acidification by bafilomycin A₁ caused a 50% reduction in the levels of transfection, independently of the lipid/DNA charge ratio used for the HSA-lipoplexes. It should be noted that none of these treatments had any toxic effect on the cells, as we have reported elsewhere [18].

To explore the possible existence of specific receptors for HSA that might facilitate the uptake of HSA-lipoplexes, competitive inhibition studies were also performed. For this purpose, COS-7 cells were preincubated with 8 mg of free HSA/0.3 ml of DME-HG medium for 30 min at 37°C, before the HSA-lipoplexes were added to the cells and incubated for 1 h. The medium was then replaced with DME-HG containing 10% FBS, and the cells were further incubated for 48 h at which time they were harvested for luciferase activity measurements. The presence of a large excess of free HSA in the medium, representing 250 times the amount of HSA associated with lipoplexes, had no significant effect on the levels of transfection mediated by HSA-lipoplexes at the 1/1 (+/−) charge ratio (Fig. 5).

3.6. Transfection by HSA-lipoplexes in vivo

To examine the potential use of HSA-lipoplexes for in vivo gene therapy applications, we determined luciferase gene expression in the lungs and spleen of mice following intravenous administration. Plain lipoplexes and naked DNA were used as controls. HSA-lipoplexes mediated significantly higher levels of luciferase gene expression in the lungs and spleen compared to plain lipoplexes or naked DNA (Fig. 6).

4. Discussion

Our results demonstrate that the association of HSA with cationic liposomes prior to the complexation of DNA produces a ternary complex that has much higher transfection activity and efficiency than plain lipoplexes at various (+/−) charge ratios. The enhancement is evident not only in readily transfected adherent cell lines, but also in lymphocytic cell lines and primary macrophages that are not transfected to an appreciable extent with plain lipoplexes. It is surprising that the major protein component of serum, which is usually inhibitory to lipoplex-mediated transfection, especially for highly positively charged complexes [33,34,44], would enhance transfection when associated with cationic liposomes before complexation with plasmid DNA.

Although the exact mechanism of the enhancement of gene delivery and expression by HSA-lipoplexes is not known, our experiments indicate that the endocytotic pathway is involved. HSA is thought not to interact with human cells through a specific cell surface receptor [19]. Thus, the enhancement of gene expression by HSA-lipoplexes is not likely to be via
binding to a specific receptor, unlike the mechanism proposed previously for transferrin-lipoplexes [15]. Our experiments showing that excess free HSA does not inhibit transfection support this hypothesis. Nevertheless, the fact that only a partial inhibition of transfection by cytochalasin B (an inhibitor of non-coated pit-mediated endocytosis) was observed, and the finding of a more extensive inhibition by metabolic inhibitors (which inhibit both coated pit and non-coated pit endocytosis) suggest that some of the internalization of HSA-lipoplexes is via coated pit-mediated endocytosis. It is therefore likely that the HSA-lipoplexes bind non-specifically to cell surface receptors, analogous to scavenger receptors, which in turn mediate their endocytosis. Conjugation of drugs or sugar residues to albumin results in an efficient drug carrier system exhibiting selective targeting to different types of cells in the liver [45,46]. Similar results were reported for anionized albumins obtained through succinylation or aconitylation of the protein. These results suggested that the uptake of these neoglycoproteins by endothelial cells or macrophages is mediated by sugar-specific or scavenger receptors [45,46]. The net negative charge exhibited by the most active HSA-lipoplexes supports the hypothesis that a scavenger receptor-like receptor may be involved.

Experiments utilizing baflomycin A1 indicate that the acidification of endosomes plays a partial role in transfection facilitated by HSA. Albumin has been described as being able to undergo a low pH-induced conformational change, thereby acquiring fusogenic properties [20,47,48]. Thus, the partial protonation of HSA at endosomal pH and its subsequent interaction with the endosome membrane may be involved in the destabilization of the latter. This destabilization may then promote the transbilayer movement (flip-flop) of anionic lipids from the cytoplasmic leaflet of the endosome membrane to the lumenal leaflet. The anionic lipids may then mediate the dissociation of the lipoplex and the entry of DNA into the cytoplasm, as suggested by Xu and Szoka [49]. It is also possible that the conformational change of albumin, involving a reversible expansion of the protein [47], reinforces the dissociation of the complexes promoted by lipid flip-flop. Experiments utilizing ANS as a fluorescent probe have indicated that albumin associated with cationic liposomes and DNA exposes hydrophobic domains under acidic conditions (unpublished data), which may result in dissociation of the complexes and promote destabilization of the endosomal membrane.

Highly positively charged complexes have been proposed to be more stable in the biological milieu, since an excess of positive charge may result in a more compact or condensed complex, enabling better protection of the DNA molecule against nucleases [50]. However, for the same reasons (i.e. more stable or compact DNA), a decrease of transfection activity may also occur (as obtained with the 2/l lipid/DNA complexes in this study) due to the difficulty of dissociation of DNA from the complex and its subsequent release into the cytoplasm.

Our in vitro observations indicate that the complexes (either net negatively or positively charged, or neutral) remain effective despite any possible interaction with serum components. The fact that transfection mediated by the HSA-lipoplexes is not affected by the presence of serum not only results in a simplification of the transfection procedure, since the washing steps can be eliminated, but also increases the possibility that these complexes can be utilized for gene delivery in vivo. Our in vivo data indeed indicate that HSA-lipoplexes are much more effective than plain lipoplexes (Fig. 6). It is likely that the presence of a major component of serum, HSA, on the lipoplexes minimizes their interaction with other serum components, including oleic acid and heparin which were shown to promote the dissociation of genetic material from the complexes [14]. Additional advantages of HSA are that it is an abundant protein, is easy to prepare and purify, and is not expected to be immunogenic.

The association of HSA and fusogenic peptides with lipoplexes may overcome some of the limitations associated with the use of cationic liposomes in gene therapy. Indeed, the ternary complexes of cationic liposomes, DNA, and HSA, not only lead to high levels of transfection, but also have the advantages of being active in the presence of serum and being non-toxic. Such ternary complexes are also likely to alleviate the problems associated with the use of highly positively charged complexes in vivo, such as avid complexation with serum proteins. These ternary complexes, and their future derivatives, such as those utilizing serum-resistant sterically
stabilized cationic liposomes, may thus be potential alternatives to viral vectors for gene delivery in vivo.

Acknowledgements

We thank Elizabeth Pretzer for the preparation of the macrophages used in this project and to Henrique Faneca for his help with the zeta potential determinations. This work was supported by (1) funds from the University of the Pacific School of Dentistry, (2) JNICT, PRAXIS XXI (BD 4056/94) and PRAXIS/PCNA/BIO/45/96 (Portugal), and (3) Grant BIO4-CT97-2191 from the European Union.

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