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ORIGINAL ARTICLE

The Mechanism of High Transfection Efficiency of Human Serum Albumin Conjugated Polyethylenimine in A549 Cells

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Background: In our previous study, HSA-PEI demonstrated high pDNA transfection efficiency and low cytotoxicity. **Materials and Methods:** In this study, the relationship between albumin receptors and the high pDNA transfection efficiency of HSA-PEI was investigated by fluorescent microscopy and flow-cytometer in A549 cells. **Results:** According to our results, the presence of an albumin receptor on A549 cells was confirmed via the application of a fluorescent tracer, FITC-HSA, and the dose-dependent competition of HSA. The transfection efficiency of HSA-PEI/pEGFP showed a dose-dependent inhibition when different amounts of HSA were added to the culture medium of the A549 cells. However, the inhibitory effect of HSA did not affect the transfection efficiency of some cationic transfection enhancement reagents, such as lipofectamine, jetPEI-RGD or PEI; their transfection was a result of contact between the positively charged reagents and the negatively charged cell surface. **Conclusion:** It was determined that, unlike other cationic reagents, the high transfection efficiency of HSA-PEI was not from the electronic effect but, instead, predominantly from its ligand effect.

Key words: Human serum albumin conjugated polyethylenimine, plasmid DNA, transfection

INTRODUCTION

Polyethylenimine (PEI) is widely used in DNA transfection because its intense positive charge spontaneously condenses the negatively charged plasmid DNA (pDNA) attached to the cell membrane, subsequently triggering cell endocytosis.[1,2] Unfortunately, the cytotoxicity of PEI has limited its application.[3]

GP-60, a 60 kDa glycoprotein present in alveolar epithelial cells, and other albodin-like proteins located in the alveoli showed evidence of albumin transportation.[4,5] Tiruppathi *et al*.[6] reported that GP-60 activation mediates albumin transcytosis in endothelial cells via the tyrosine kinase-dependent pathway. From these reports, it can be concluded that albumin receptors exist in the epithelial and endothelial cells of the respiratory system in order to transport albumin.

Human serum albumin (HSA) is a human endogenous protein with a molecular mass 67 kDa that is produced in the liver. The researchers chose to apply HSA to the pDNA delivery systems due to its possible candidacy as a ligand in the respiratory system, its low toxicity, low inflammatory effect and low cost. Carrabino *et al*. (2005)[8] reported that HSA enhanced pDNA transportation via physical mixing of the PEI/pDNA complex. In order to increase the fixation of HSA, chemical cross-linkage methods were also used to conjugate HSA to the surface of the PEI/pDNA complex.[9] Chen *et al*. (2014)[10] reported that if HSA was chemically conjugated with PEI (HSA-PEI), the cytotoxicity of PEI could be reduced. In their article, the characteristics of HSA-PEI were presented, including: Fourier transform infrared spectrometry (FTIR) and Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) spectrograms, size, zeta potential, plasmid enhanced green fluorescent protein (pEGFP) bonding and protecting efficiency, as well as evidence of high pDNA transfection efficiency and low cytotoxicity.

The mechanism of the high pDNA transfection efficiency of HSA-PEI was unclear. The main objective of this study, therefore, was to investigate the relationship between albumin receptors and the high pDNA transfection efficiency of HSA-PEI.

MATERIALS AND METHODS

Materials

Human serum albumin (HSA, fraction V, 65 kDa), PEI, branched, average MW ~25,000 by LS, average Mn ~10,000 by GPC, N-ethyl-N′hyl-gedleniminopropyl) carbodiimide

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hydrochloride (EDAC, EDC hydrochloride), and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). JetPEI-RGD (Polyplus, Illkirch, France) and lipofectamine (Invitrogen, Kaohsiung, Taiwan) were commercially available products. Model plasmid, pEGFP-N1 (Catalog number 6085-1, Clontech Laboratories, Inc., Taiwan Office, Taipei, Taiwan), was encoded with GFP gene. All reagents were of analytical or reagent grade.

Cell lines and culture

A549 cells (adenocarcinomic human alveolar basal epithelial cell line, ATCC CCL-185) were cultured using DMEM/F12 medium (Gibco Invitrogen Corporation, Grand Island, New York, USA) supplemented by 10% fetal bovine serum (FBS) and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Synthesis of human serum albumin-polyethylenimine

Zero-length linker EDC hydrochloride was used to connect HSA and PEI, as in our previous study.[9]

Confirmation of albumin receptors on cells

Fluorescein isothiocyanate-human serum albumin was prepared following the protocol of Sigma CAS#: 3326-32-7 FITC Data Sheet. A549 cells (adenocarcinomic human alveolar basal epithelial cells with albumin GP-60 receptor) were seeded on a 24-well culture plate with a density of 1×10^5 cell/well, and cultured in DMEM/F-12 medium containing 10% FBS at 37°C and 5% CO_2 for 24 h. The cells were washed by phosphate-buffered saline (PBS) 1 time. The medium was replaced by 900 μL of serum-free DMEM/F-12 medium. 100 μL of 10 μM FITC-HSA was added, and the cells were cultured for a further 4 h. The cells were then washed by PBS 2 times. Fluorescent microscopy (IX-70, Olympus, Tokyo, Japan) was used to evaluate the adhesion of HSA-PEI to cells.

Competition of human serum albumin and fluorescein isothiocyanate-HSA on cells

A549 cells were seeded on a 6-well culture plate with a density 2×10^5 cell/well, and cultured in DMEM/F-12 medium containing 10% FBS at 37°C and 5% CO_2 for 24 h. The cells were washed by PBS 1 time. The medium was replaced by 2.5 mL of serum-free DMEM/F-12 medium, which contained 0, 15, 30, 150 or 300 mg of HSA and then incubated for another 3 h. Finally, 500 μL of serum-free DMEM/F-12 medium containing 30 μM FITC-HSA was added and cells were cultured for 1-h. The final medium contained 0, 5, 10, 50 or 100 hA mg/mL and 10 μM/mL FITC-HSA in 3 mL of medium. The cells were washed by PBS 2 times. A flow-cytometer (FACS Calibur, Becton, Dickison, USA) with a 488 nm laser beam was used to evaluate the adhesion of FITC-HSA to cells.

Effect of human serum albumin on the transfection of HSA-polyethylenimine/plasmid enhanced green fluorescent protein

30 μg HSA-PEI and 30 μg pEGFP were separately prepared in 1 mL of serum-free DMEM/F-12 medium. After mixing, via a gentle shaking for 30 s, the nanoscaled HSA-PEI/pEGFP w/w 1 was formed.

A549 cells were seeded on a 6-well culture plate with a density of 2×10^5 cell/well and cultured in DMEM/F-12 medium containing 10% FBS at 37°C and 5% CO_2 for 24 h. The cells were washed by PBS 1 time. The medium was replaced by 2 mL of serum-free DMEM/F-12 medium. 500 μL of serum-free DMEM/F-12 medium containing 0, 3, 30 or 150 mg/mL of HSA was also added and cells were incubated for 30 min. A volume of 500 μL of serum-free DMEM/F-12 medium containing 30 μg of HSA-PEI/pEGFP w/w 1 was then added (final medium contained 0, 1, 10 or 50 hA mg/mL and 10 μM/mL FITC-HSA in 3 mL of medium) and cells were cultured for another 3.5 h. Finally, the medium was replaced by 3 mL of DMEM/F-12 medium containing 10% FBS and incubated for 44 h. The cells were washed by PBS 2 times. A flow-cytometer (FACS Calibur, Becton, Dickison, USA) with a 488 nm laser beam and a fluorescence microscope (IX-70, Olympus, Tokyo, Japan) were used to evaluate the transfection of HSA-PEI/pEGFP. In this study, lipofectamine v/w 1, jetPEI-RGD v/w 1 and PEI w/w 1 were used as control groups.

Effect of 1% medium serum on the transfection of human serum albumin-polyethylenimine/plasmid enhanced green fluorescent protein

This experiment was carried out using the same procedures outlined above but the serum-free medium with/without HSA was replaced by medium containing 1% FBS.

STATISTICS

Statistical significance was evaluated by unpaired Student's *t*-tests.

RESULTS

Checking of albumin receptor on A549 cells

In order to check that the albumin receptor existed on the A549 cells used in this study, an albumin fluorescent tracer, FITC-HSA, was prepared as per the FITC supplier's protocol. Figure 1 shows that the fluorescent tracer attached to A549 cells.

Figure 2a demonstrates that the fluorescence on the A549 cells was a result of the conjugated FITC-HSA attachment and not from unconjugated HSA or FITC. Figure 2b shows that the binding of HSA-PEI on A549 cells has a tolerance of 5 mg/mL HSA; when the concentration of HSA was >10 mg/mL, there was a dose-dependent competition with the environmental HSA.

Occupation of human serum albumin in the binding-site of HSA-polyethylenimine on A549 cells

HSA-PEI/pEGFP had been shown to provide better pEGFP transfection efficiency than PEI/pEGFP, jetPEI/pEGFP or lipofection. In order to evaluate whether the effect of HSA-PEI was related to the albumin receptor or not, A549 cells were treated with HSA, ranging from 1 to 50 mg/mL. HSA-PEI/pEGFP transfection tests were then carried out and evaluated by flow-cytometry and fluorescent microscopy, as shown in Figures 3 and 4 respectively. The results showed that the transfection efficiency of HSA-PEI/pEGFP was dose-dependent and strongly affected by HSA occupying the binding-site.

Significance of binding-site occupation on human serum albumin effect

In order to evaluate whether the effect of HSA was due to its binding-site occupation, the same experiment was repeated using lipofectamine, jetPEI-RGD and PEI w/w 1. The results are shown in Figure 5 and demonstrate that 10 mg/mL of HSA did not affect the transfection of lipofectamine/pEGFP, jetPEI-RGD/pEGFP or PEI/pEGFP.

Feasibility of human serum albumin-polyethylenimine in a low serum situation

In order to evaluate whether HSA-PEI could be used in a low serum situation, 1% FBS was added to the transfection process.

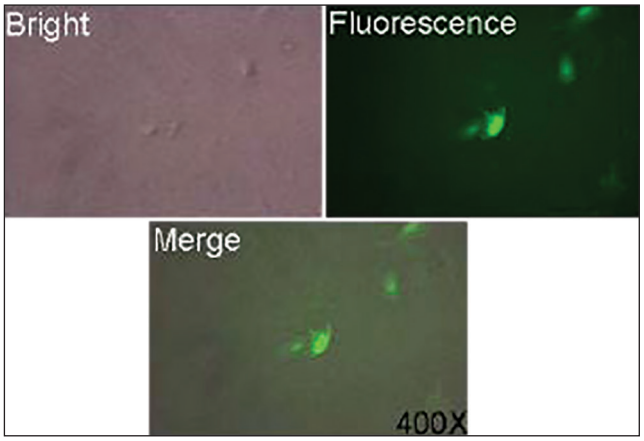

Figure 1. Microscopic photographs of fluorescein isothiocyanate-human serum albumin (FITC-HSA) attached to A549 cells. A fluorescent tracer, FITC-HSA, was attached to A549 cells

The results [Figure 6] show that the transfection of lipofectamine/pEGFP was reduced 14%, but the other groups, including HSA-PEI, jetPEI-RGD and PEI, were not obviously affected.

DISCUSSION

Albumin is a crucial protein in blood and tissue fluid. There are many epithelial and endothelial cells composed of albumin-related receptors (such as GP-60, a 60 kD albumin receptor). Tiruppathi *et al*.[6] proved that GP-60 in bovine pulmonary microvessel endothelial cells mediated albumin transcytosis via the tyrosine kinase-dependent pathway. John *et al*.[4] reported the role of GP-60 in active transalveolar albumin transport in isolated lung type II epithelial cells in rats. Kim *et al*.[5] demonstrated the absorption of FITC labeled bovine serum albumin across primary cultured rat alveolar epithelial cells. This evidence suggests the albumin receptor as a possible pathway to deliver therapeutic material.

A549 cells are derived from a human adenocarcinomic alveolar basal epithelial cell line. Yumoto *et al*.[7] proved the existence of albumin receptors on A549 cells. In this study, the existence of albumin-related receptors on the A549 ATCC CCL-185 strain was

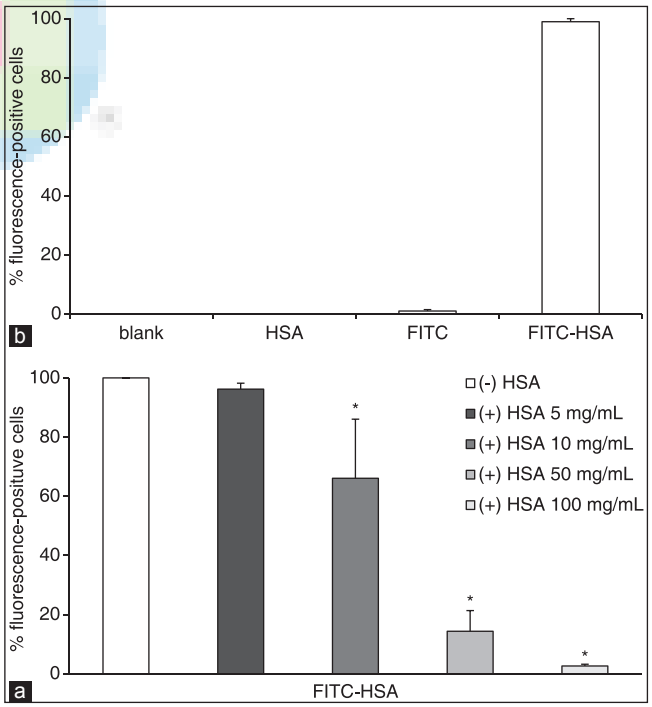

Figure 2. Receptor binding assay of fluorescein isothiocyanate-human serum albumin (FITC-HSA) (n = 3). (a) Percentage of fluorescence-positive cells. (b) Effect of HSA on the binding of FITC-HSA. The fluorescent spots shown in microscopic photographs of the A549 cells were a result of the binding of FITC-HSA, rather than from FITC or HSA alone, as shown in Figure 2a. The binding of FITC-HSA was subject to dose-dependent competition with HSA, as shown in Figure 2b

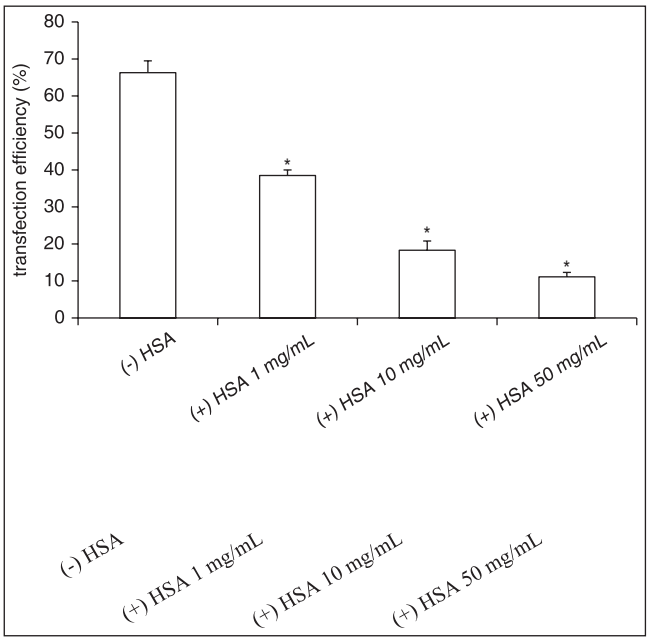

Figure 3. Effects of human serum albumin (HSA) on the transfection of HSA-polyethylenimine (PEI)/plasmid enhanced green fluorescent protein (pEGFP), as observed via flow-cytometry (n = 3). HSA-PEI/pEGFP had a high transfection efficiency in A549 cells but was also subject to dose-dependent inhibition by HSA

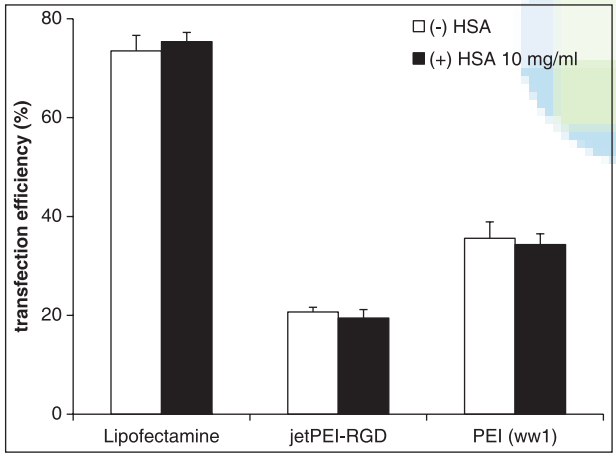

Figure 5. Effects of human serum albumin (HSA) on the transfection of cationic transfection reagents/plasmid enhanced green fluorescent protein (pEGFP), as observed by flow-cytometry (n = 3). The mechanism of the transfection of cationic transfection reagents/pEGFP was predominantly from contact between the high-density cationic and anionic cell surfaces, which subsequently induced cell endocytosis. However, the addition of HSA did not affect the transfection of cationic transfection reagents/pEGFP

confirmed by the use of FITC labeled HSA [Figure 1]. The binding-site of FITC-HSA was subject to dose-dependent competition and occupation by HSA, as shown in Figures 2a and b.

The ligand-binding properties of albumin may potentially be used to deliver therapeutic material. Carrabino *et al*.[8] mixed HSA with PEI/pDNA complexes on A549 and 9HTEo cells to increase

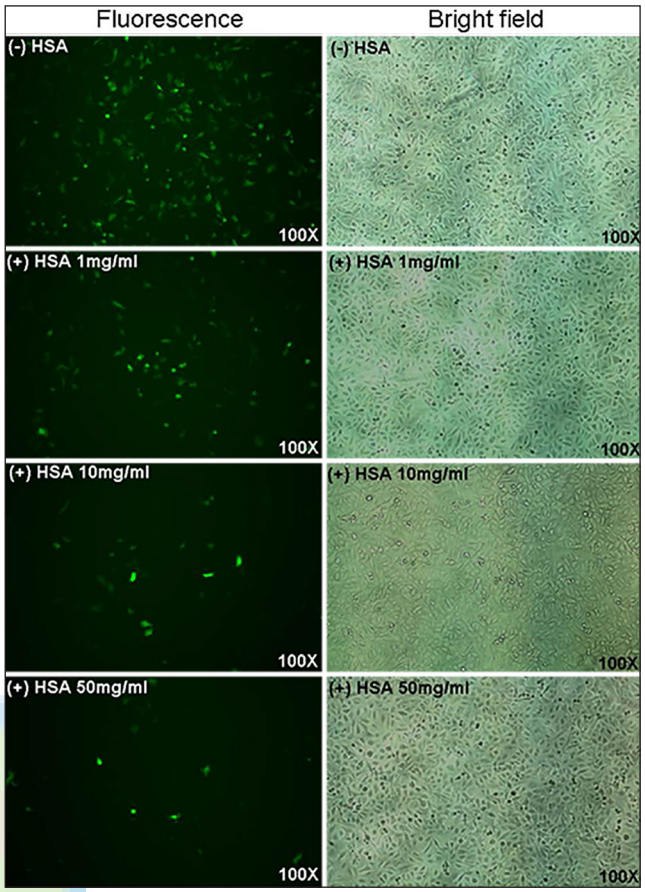

Figure 4. Effects of human serum albumin (HSA) on the transfection of HSA-polyethylenimine/plasmid enhanced green fluorescent protein, as observed via fluorescent microscopy

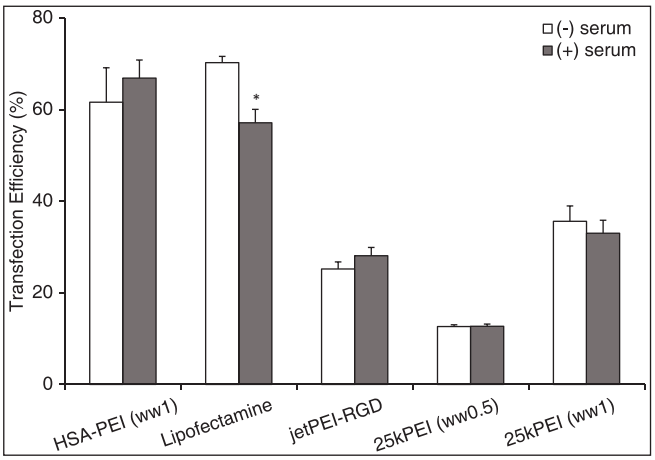

Figure 6. The transfection of human serum albumin (HSA)-polyethylenimine (PEI)/plasmid enhanced green fluorescent protein (pEGFP) in low serum conditions (n = 3). A culture medium containing 1% fetal bovine serum had no effect on the transfection efficiency of HSA-PEI/pEGFP

the transfection efficiency of model plasmid pEGFP. Rhaese *et al*.[9] prepared a pGL3 loaded HSA nanoparticle by mixing

2.6-17 μg PEI, 10 μg pDNA and 10 mg HSA that were then cross-linked by EDAC. The nanoparticle was applied to HEK 293 cells to increase the transfection efficiency of model plasmid pGL3. HSA was chemically conjugated with PEI (HSA-PEI). Chen *et al*. reported that HSA-PEI showed good pDNA delivery properties including: Low cytotoxicity, high transfection efficiency, good pDNA protection and ease of use via simple one-step mixing with pDNA to form a nano-sized complex.[10] In this study, more of HSA-PEI advanced characteristics were examined. Figures 3-5 demonstrate the interaction between HSA-PEI and the albumin receptor, ultimately eliciting the mechanism responsible for high HSA-PEI/pDNA transfection efficiency. The pretreatment of HSA significantly reduced the pEGFP transfection efficiency of HSA-PEI/pEGFP (as shown in Figures 3 and 4) but did not affect the transfection of lipofectamine, jetPEI-RGD or PEI (as shown in Figure 5). Lipofectamine, jetPEI-RGD and PEI were shown to be polycationic transfection vectors via nonspecific electrostatic interaction.[11,12] In addition, jetPEI-RGD was integrin receptor-mediated rather than albumin receptor-mediated. This proved the absorption of HSA-PEI was mediated by albumin receptors.

In low serum conditions, the transfection efficiency of HSA-PEI/pEGFP was not influenced by the application of a 1% FBS transfection medium, as shown in Figure 6. There was, however, an interesting find in Figures 5 and 6. Medium containing 0.17% HSA didn't affect the transfection of lipofectamine (as per Figure 5: 0.5 mL, 10 mg/mL HSA in 3 mL medium, final HSA concentration was about 0.17%), however, 1% FBS in the medium did have an effect (total protein of FBS was 4%, 1% FBS in the medium was close to 0.04% total medium protein). An unknown protein in FBS had possibly affected the efficiency of lipofectamine. There are some environmental factors that affect the efficiency of transfection reagents. Hanzlíková *et al*.[2] reported that the cell-surface glycosaminoglycans can bind with PEI and hinder its efficiency. Furthermore, the protein content of tissue fluid is about a quarter that of blood and the flow rate in tissue fluid is also much lower than that in veins. Finally, the application of HSA-PEI is currently limited to IV injection but may possibly be administered by inhalant or subcutaneous means in the future.

CONCLUSION

In this study, the high transfection efficiency of HSA-PEI was shown to be dependent on albumin receptor mediation. Our results may be useful in deciding the best administration route in future animal studies.

ACKNOWLEDGMENTS

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