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Anionic Liposomes Increase the Efficiency of Adenovirus-Mediated Gene Transfer to Coxsackie-Adenovirus Receptor Deficient Cells

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Abstract: Despite remarkable progress in the research of both viral and nonviral gene delivery vectors, the drawbacks in each delivery system have limited their clinical applications. Therefore, one of the concepts for developing novel vectors is to overcome the limitations of individual vectors by combining them. In the current study, adenoviral vectors were formulated with anionic liposomes to protect them from neutralizing antibodies and to improve their transduction efficiency in Coxsackievirus-adenovirus receptor (CAR) deficient cells. A calcium-induced phase change method was applied to encapsulate adenovirus 5 (Ad5) into anionic liposomes to formulate the complexes of Ad5 and anionic liposomes (Ad5−AL). Meanwhile, the complexes of Ad5 and cationic liposomes (Ad5−CL) were also prepared as controls. LacZ gene expression in CAR overexpressing cells (A549) and CAR deficient cells (CHO and MDCK) was measured by either qualitative or quantitative detection. Confocal laser scanning microscopy was performed to determine intracellular location of Ad5 after their infection. Human sera with a high titer of antiadenovirus antibody were used to assess the neutralizing antibody protection ability of the complexed vectors. Accompanying the enhanced gene expression, a high ability to introduce Ad5 into cytoplasm and nucleus mediated by Ad5−AL was also observed in CAR deficient cells. Additionally, antibody neutralizing assay indicated that neutralizing serum inhibited naked Ad5 and Ad5−CL at rather higher dilution than Ad5−AL, which demonstrated Ad5−AL was more capable of protecting Ad5 from neutralizing than Ad5−CL. In conclusion, anionic liposomes prepared by the calcium-induced phase change method could significantly enhance the transduction ability of Ad5 in CAR deficient cells.

Keywords: Gene therapy; adenovirus 5; anionic liposomes; encapsulation; CAR; antibody neutralization

Introduction

A safe and efficient gene transfer system is the focus in gene therapy studies nowadays. Although a wide variety of vectors for gene transfer have been used for experimental purposes, they can be classified into viral and nonviral delivery systems. Among viral vectors, adenoviruses can infect virtually many cell types in either dividing or nondividing periods[1−3] and have been widely used as vectors for gene therapy and genetic vaccine. But adenoviral vectors also have several limitations. The uptake of adenovirus is

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dependent on the expression of Coxsackievirus-adenovirus receptor (CAR) on the cell surface.[4] Some cell lines are relatively resistant to adenovirus transduction for lack of CAR, and, consequently, high doses of virus and long periods of physical contact between the vector and target cells are required for efficient gene transfer. However, high doses of virus could result in viral precipitation and local inflammation that may present a substantial risk to the patient.[5,6] Moreover, extensive vector contact with cells such as the airway epithelium is prevented by natural clearance mechanisms as well as mucous and soluble factors in airway surface fluid.[7,8]

On the other hand, nonviral delivery systems have been increasingly proposed as safer alternatives to viral vectors. They have the potential to be administered repeatedly with minimal host immune response, they keep stable in storage, and they are easy to produce in large quantities.[9−11] These advantages have provided the impetus to continue their development. Nevertheless, during nonviral gene transfer, entry of exogenous DNA into the nucleus occurs only in cells that are actively dividing, i.e., when the nuclear envelope breaks down. Pollard et al. showed that less than 1/1000 naked cDNA copies microinjected in the cytoplasm were effectively trafficked to the nucleus.[12] This leads to the result of low gene transfer efficiency by nonviral delivery systems.

In a word, many viral and nonviral methods of gene transfer have been developed, but each has its limitations and advantages. Thus, one of the strategies for the development of novel vectors is to overcome the limitations of individual vectors by combining them.

A series of attempts[13−15] have been made in using cationic liposomes to redirect tissue tropism of adenovirus and overcome the immune barrier. In Ma's study, they reported that preinjection of cationic liposomes followed by adenovirus led to a significant increase in the level of gene expression in the lung.[14] Also, Han's study demonstrated that limitations of gene therapy approaches involving the use of adenoviral vectors due to the CAR deficiency of target cells and liver toxicity could be overcome by complexing the adenovirus with cationic liposomes.[15] These results were exciting for the application of adenovirus in gene therapy. However, the utility of cationic liposomes in gene therapy is hampered by toxicities of the cationic lipids, limited *in vivo* gene transfer efficiency and low tissue specificity. These shortcomings may be due to poor colloidal stability and incompatibility with the abundance of negatively charged macromolecules present in the physiological environment. To overcome the disadvantages of cationic liposomes, adenoviral vectors were encapsulated into anionic liposomes in the current study. As naturally occurring components in eukaryotic cell membranes, anionic lipids have been examined as alternatives to cationic liposomes in the delivery systems of plasmid DNA, because of their low cytotoxicity.[16−18] However, their overall efficiencies in DNA delivery are much lower than those of their cationic counterparts. Distinguished from previous reports to formulate plasmid DNA with anionic liposomes, we, for the first time, successfully encapsulated adenoviral vectors into anionic liposomes by

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a calcium-induced phase change method forming the complexes of Ad5 and anionic liposomes (Ad5−AL). As a control, the complexes of Ad5 and cationic liposomes (Ad5−CL) were also prepared. The cytotoxicities, transduction efficiencies in CAR deficient cells and protection abilities from neutralizing antibodies of both Ad5−AL and Ad5−CL were investigated and compared.

Materials and Methods

Virus Preparation and Titer Determination. Recombinant adenovirus vectors (Ad5) expressing LacZ reporter gene, E1/E3-deleted replication-deficient, were purchased from the VGTC Gene Technology Company Ltd. Ad5 was propagated in HEK 293 cells (ATCC CRL1573) cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin. Infection of HEK 293 cells was carried out in DMEM supplemented with 2% FBS for the first 24 h, after which FBS was added to bring the final concentration to 10%. Infected cells were harvested when 100% of the cells exhibited virus induced cytopathic effect, collected, and concentrated by centrifugation at 2000*g* for 10 min at 4 °C. Cell pellets were resuspended in 10 mM Tris (pH 8.0) and lysed by three cycles of freezing and thawing. Virus preparations were obtained following two ultracentrifugation steps on cesium chloride (Amersco, American) gradients and were desalted by dialysis at 4 °C against a mixture of 10 mM Tris (pH 8.0), 2 mM $MgCl_2$, 5% sucrose. Stocks of virus were aliquoted and frozen at −80 °C.

The titer of virus preparations was determined by Taqman real-time PCR[19] and plaque assay,[20] respectively. Plaque assays were performed in duplicate. The final plaque count was determined on day 14. For the determination of the genome copy number of stock adenovirus, the forward primer (CAAAGACTGGTTCCTGGTACAAATG), reverse primer (GTGTAGGATGCCCACCTGTTG) and the probe (5′-FAM AGTATCATCCACCACCTGACGGCTCBHQ1-3′) were designed and synthesized according to a conserved region of the species C adenovirus hexon gene (nucleotides 21127 to 21304 of Ad5; GenBank accession number NC_001406). Viral nucleic acids were obtained from viral stocks using the High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland). The real-time PCR amplification was carried out in 25 μL reaction mixtures consisting of 10 μL iQ supermix (Bio-Rad), 250 nM each primer, 250 nM TaqMan probe and 1 μL of DNA template. Thermocycling profiles for it consisted of 1 cycle of 95 °C for 3 min followed by 45 cycles of 95 °C for 15 s, 54.3 °C for 20 s and 72 °C for 10 s in a Bio-Rad iQ5. This amplification yields a 177 bp product.

Preparation of Complexes of Adenovirus with Liposomes. A negatively charged cholesterol derivant, cholesteryl-hemisuccinate (CHEMS), *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (Tes), dioleoylphosphatidyl ethanolamine (DOPE) and 3β-*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl cholesterol (DC-Chol) were purchased from Sigma-Aldrich Corp (St. Louis, MO). Egg phosphatidylcholine (PC) was from Doosan Korea; cholesterol and ethylenediaminetetraacetic acid (EDTA-disodium salt) were from Amersco.

Blank anionic liposomes composed of CHEMS/PC/Chol (4:5:1, molar ratio) and blank cationic liposomes composed of DC-Chol/DOPE (1:1, molar ratio) were prepared by the thin film hydration and sonication dispersion technique.[21,22]

The complexes of naked adenovirus and anionic liposomes (Ad5−AL) were formed based on the calcium-induced phase change method.[23] Ca^{2+} was introduced either by direct addition of 100 mM $CaC1_2$ to the blank anionic liposomes (final concentration 10 mM) and incubation at 37 °C for 1 h or by dialysis against buffer containing 100 mM NaCl, 2 mM histidine, 2 mM Tes, and 1.5 mM $CaC1_2$ at pH 7.4 overnight at room temperature. The resulting precipitate was pelleted by centrifuging at 2500*g* for 10 min. The pellet was resuspended at a lipid concentration of 10 μmol/mL in a concentrated virus solution or Tes buffer by vortex mixing for 10 min at room temperature. EDTA (100 mM) was added directly to this solution with a final concentration of 15 mM, and 10−20 μL of 100 mM NaOH was added to adjust the pH to 7.4. The solution was vortex mixed for 10 min at 37 °C followed by incubation for 30 min. All buffers and solutions were sterilized by filtration or autoclaving prior to addition to vesicle preparations.

For preparing the complexes of Ad5 and cationic liposomes (Ad5−CL), the blank cationic liposomes with varying lipid concentrations according to the need of following gene transfer studies were added to an equal volume of Ad5−LacZ and incubated at room temperature for 15 to 30 min prior to use.

Transmission Electron Microscopy. Naked adenovirus and complexes of anionic liposome with adenovirus particles were processed for transmission electron microscopy (TEM) using a negative stain technique. The samples were brought on Formvar/carbon coated 400 mesh grids made hydrophilic by glow discharge in air directly. After 3 min, the sample solution was removed by gentle blotting with filter paper

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and stained with uranyl acetate (1% w/v) for 30 s. The samples were air-dried for 15 min to completely remove the water, and the infrared light was used for drying when necessary. Afterward, the samples were examined by a Jeol JEM-1400 electron microscope operated at 80 kV.

Photon Correlation Spectroscopy and Determination of Zeta Potential. The average diameter and polydispersity index of complexes were measured by photon correlation spectroscopy (PCS) (Malvern Zetasizer Nano ZS90, Malvern Instruments Ltd., U.K.) with a 50 mV laser. Typically, 0.2 mL of samples was diluted by 1 mL of water before adding into the sample cell. The measurements were performed at 25 °C at a fixed angle of 90°. The measurement time was 2 min, and each run underwent 10 subruns. Each value reported is the average of at least three measurements.

Zeta potential of the lipid carriers was measured by Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd., U.K.). Before the measurements, the samples were diluted 1:5 in distilled water. Each sample was analyzed in triplicate, and the zeta potential was derived from mobility of particles in electric field by applying the Smoluchowsky relationship.

Cell Viability Assay. A549 cells (1×10^4) were seeded into 96-well plates one day before being treated with 100 μL of different formulations. The naked Ad5, the Ad5−AL and the Ad5−CL (at total lipids 400, 200, 100 μg/mL) were prepared in DMEM respectively with the Ad5 at a variety multiplicity of infection (MOI) of 80, 40, and 20 pfu/cell. After 4 h incubation, 200 μL of DMEM complete media was added to each well, and cells were cultured for 24 h. Cell viability was determined by MTT assay,[24] and it was calculated according to the formula $(A_{\text{treated}} - A_{\text{background}}) \times 100/(A_{\text{control}} - A_{\text{background}})$, in which the control cells were not exposed to samples and the background well had no cells in it. It was completed in triplicate.

Cell Culture and Gene Transduction. A549 (ATCC CCL-185), CHO (ATCC CCL-61) and MDCK (ATCC CCL-34) cells were grown in DMEM supplemented with 10% newborn calf serum, 2% penicillin/streptomycin, and 1% L-glutamine and maintained at 37 °C and 5% CO_2. For gene transfer, A549, CHO and MDCK cells were seeded at 2.5×10^5 cells/well in 24-well plates and grown to confluency. Prior to transduction, wells were examined under the light microscope for general uniformity and health. Then cells from at least one well were harvested, and a cell count was performed using a hemacytometer. Three kinds of formulations, naked Ad5, Ad5−AL and Ad5−CL (total lipid 100 μg/mL), were prepared in DMEM at a variety MOI of 5, 20, 40 pfu/cell, and then 300 μL/well of the transduction sample was added dropwise and incubated with the cells for 4 h at 37 °C. The transduction medium was removed after 4 h, and the cells were washed once and replaced with fresh serum containing DMEM complete medium. Cells were assayed for gene expression after 24 h by microscopic LacZ histochemistry or by quantitative detection of β-galactosidase production.

***β*-Galactosidase Histochemistry on A549, MDCK and CHO Cells.** Cells were fixed with 1% formaldehyde−0.2% glutaraldehyde in 100 μL of PBS for 10 min at room temperature. After the cells were washed twice with 200 μL of PBS, they were incubated at 37 °C in 50 μL of 2.5 mM X-Gal (5-bromo-4-chloro-3-indolyl-β-galactosidase, Invitrogen, Grand Island, NY) reaction mixture containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 2 mM magnesium chloride in PBS. After 4 h of incubation, the result was observed under the inversion microscope.

Assay for *β*-Galactosidase Enzymic Activity. Average β-galactosidase activities were determined using the β-galactosidase enzyme assay system (Applied Biosystems). The total protein content of the lysates was measured by a BCA assay (Pierce, USA) using a standard of bovine serum albumin (BSA). Infectivity was quantified as pictogram of β-galactosidase (based on a standard curve for β-galactosidase activity) per milligram of total cell protein (pg/mg protein). The data represented the mean ± SD of three wells and was representative of three independent experiments.

Ad5 Neutralization Assay. Sera for the Ad5 neutralization assays were collected from healthy human donors (West China Hospital, Sichuan University, P. R. China) previously selected for the high-titer neutralizing antiadenoviral antibodies. The study was approved by the Ethics Committee of West China Medical School, Sichuan University.

A549 cells were seeded at 3×10^4 cells per well in 96-well plates one day before the neutralization assay to reach 95−100% confluence. The sera were complement-inactivated at 56 °C for 60 min before a serial doubling dilution was performed in a new 96-well tissue culture plate. The dilutions covered a range from 11.11 to 0.0055 μL of serum in a volume of 100 μL of DMEM (eventually resulting in dilutions from 1/18 to 1/36864 in an end volume of 200 μL). No serum was added to the negative controls, which resulted in the maximum LacZ gene expression. 50 μL of the naked Ad5 (MOI 20), Ad5−CL and Ad5−AL (total lipid 100 μg/mL, MOI 20) were put in 50 μL of DMEM, respectively, and the various mixtures were incubated with preformed neutralizing antiadenovirus antiserum for 1 h at 37 °C. After incubation, 200 μL of each sample was applied to A549 cells and incubated for 4 h at 37 °C for adsorption and initiation of infection. Afterward, the supernatant was removed and replaced with 200 μL of fresh DMEM with 10% newborn calf serum and further incubated at 37 °C in 5% CO_2. LacZ gene expression was measured after incubation for 24 h by X-Gal staining described above. After 4 h of staining, optical density was measured on an enzyme-linked immunosorbent assay plate reader (model 550, Biorad USA) at 490 nm. Background absorbance was determined by wells containing cells only; maximum absorbance (as control) was determined by wells with cells and samples, without serum.

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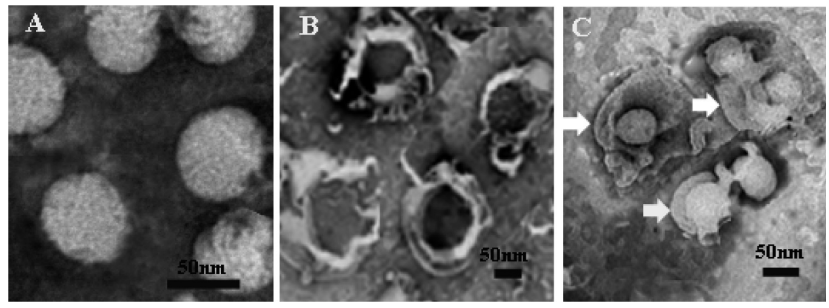

Figure 1. Electron micrographs of naked adenoviruses (A), blank anionic liposomes (B) and complexes of anionic liposomes with adenoviruses (C). The bold arrow indicates the lipid membrane. Bar represents 50 nm.

Intracellular Trafficking of the Naked Ad5 and the Complexes. *Fluorophore-Conjugated Ad5.* To permit the detection of Ad5 and liposomes without postfixation labeling procedures, the Ad5 vectors were conjugated with Alexa Fluor 555 (Molecular Probes, Invitrogen).

Alexa Fluor 555 (excitation/emission maxima ~ 555/565 nm) is a red succinimidyl ester fluorophore exhibiting almost identical fluorescence excitation and emission spectra to those commonly used of tetramethylrhodamine or Cy3 dye. Adenovirus stocks to be conjugated were adjusted to 1.0×10^{11} particles/mL and then mixed with Alexa Fluor 555 reagents previously reconstituted in dimethyl sulfoxide (DMSO, 10 mg/mL). While stirring or vortexing, a mixture of 200 μL of Ad5 preparation and 790 μL of 0.1 M sodium bicarbonate buffer (pH 8.3) was slowly mixed with 10 μL of the reactive dye solution. After 1 h incubation at room temperature, the conjugation reaction was stopped by adding 100 μL of freshly prepared 1.5 M hydroxylamine (pH 8.5). The labeled virus was separated from the excess, unconjugated dye by dialysis against a mixture of 10% glycerol, 50 mM Tris-HCl (pH 7.8), 150 mM $NaCl_2$, and 10 mM $MgCl_2$ at 4 °C overnight. The amount of dye incorporated into Ad5-labeled vectors was measured by recording absorbance at 555 and 565 nm, respectively, using a spectrophotometer (model RF-5301, Shimadzu Japan). The resulting conjugates were stored in PBS with 10% glycerol at −80 °C until use.

Intracellular Trafficking. To follow intracellular trafficking of the naked Ad5 and the complexes, A549 cells and MDCK cells were respectively seeded at 2.5×10^5 cells per well on the sterile coverslip coated with rat tail collagen in 24-well plates one day before the neutralization assay. Upon reaching 90−95% confluence, the cells were rinsed three times with PBS and infected with fluorescently labeled naked Ad5 and complexes (MOI 20) at 37 °C for 2 h. After infection, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min. Cells were then stained with DAPI (ROCHE, American) to detect the positions of nuclei and mounted on glass slides with antifade mounting medium (sigma). Cells were examined with a Leica TCS SP5 AOBS confocal microscopy system (Leica, Germany).

Statistical Analysis. Data were expressed as the means ± standard deviation of at least three independent experiments. Significant differences between sample means were calculated using Stastica, version 6.0 (StatSoft, USA) by one-way ANOVA (analysis of variance) followed by posthoc testing with Dunnet's method. Data were considered to be statistically significant if $P < 0.01$ (*) and very significant if $P < 0.001$ (**).

Table 1. Zeta Potential and Size Distribution of the Naked Adenovirus and Complexes[a]

	zeta potential (mV)	size (nm)	PDI
Ad5	−9.32 ± 1.5	93.15 ± 2	0.280 ± 0.015
blank AL	−48.8 ± 3.5	163 ± 12	0.240 ± 0.080
Ad5−AL	−41.2 ± 2.2	211 ± 10	0.268 ± 0.012
blank CL	59.4 ± 3.8	186 ± 12	0.245 ± 0.015
Ad5−CL	48.9 ± 2.9	239 ± 15	0.330 ± 0.020

[a] AL: anionic liposomes. CL: cationic liposomes. Results are expressed as the means ± SD from at least three measurements.

Results

Characterization of Adenovirus and Complexes of Adenovirus with Liposomes. The results of plaque assays and Taqman real-time PCR demonstrated that the titer of stock adenovirus is 1.33×10^{10} pfu/mL and 2.93×10^{11} genome copy /mL, respectively. The results of transmission electron microscopy are shown in Figure 1. Figure 1(A) demonstrates that the average particle size of adenovirus was about 60 nm. In Figure 1(B), the dark "core" inside of blank liposomes actually resulted from the accumulation of the staining solution used in TEM. It could be seen from Figure 1(C) that the naked adenoviruses were surrounded by lipid membranes. Additionally, in TEM sample preparation, the samples were air-dried for 15 min to completely remove the water and the infrared light was used for drying when necessary. Such a drying process apparently caused shrinkage and resulted in the roughness of the liposome surface. Also in Figure 1(C), the liposomes were not spherical due to the same reason.

The PCS results and zeta potential of the naked Ad5 and the liposomes are summarized in Table 1. Naked Ad5 exhibited a diameter of 93.15 ± 2 nm, which was larger than that determined by TEM. This may be due to these two methods being based on different mechanisms and employing different sample preparation processes, which might lead to the discrepancy of the outcomes. The size detection of adenovirus by PCS was carried out in aqueous state, and in this case, adenovirus was highly hydrated and the diameters detected by PCS were "hydrated diameters", which were usually larger than their genuine diameters. Moreover, it

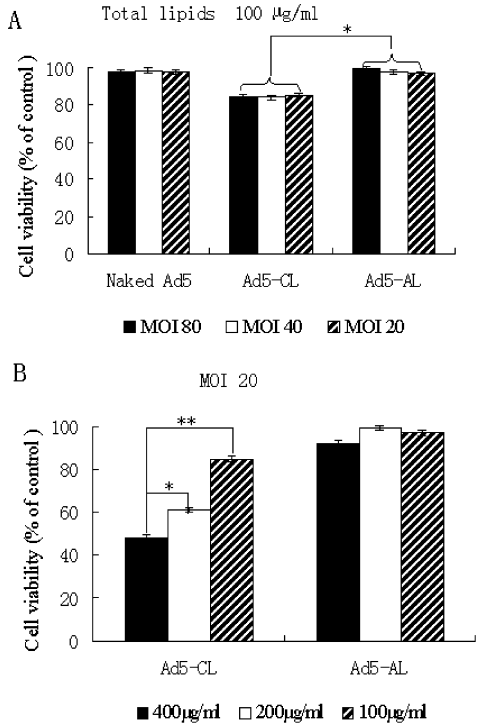

Figure 2. Viability of A549 cells determined by MTT assay. A549 cells were incubated with 50 μL of three different formulations: the naked Ad5, Ad5 complexed with anionic liposomes (Ad5−AL) and Ad5 complexed with cationic liposomes (Ad5−CL). (A) A549 cells were treated by different formulation at MOI of 80, 40, and 20 pfu/cell, respectively. The lipid concentrations in both Ad5−CL and Ad5−AL were 100 μg/mL. (B) The lipid concentrations in Ad5−CL and Ad5−AL were 400, 200, and 100 μg/mL, respectively with a MOI of 20 pfu/cell. Results are presented as mean ± standard deviation (SD) ($n = 3$), $^*P < 0.01$, $^{**}P < 0.001$, ANOVA.

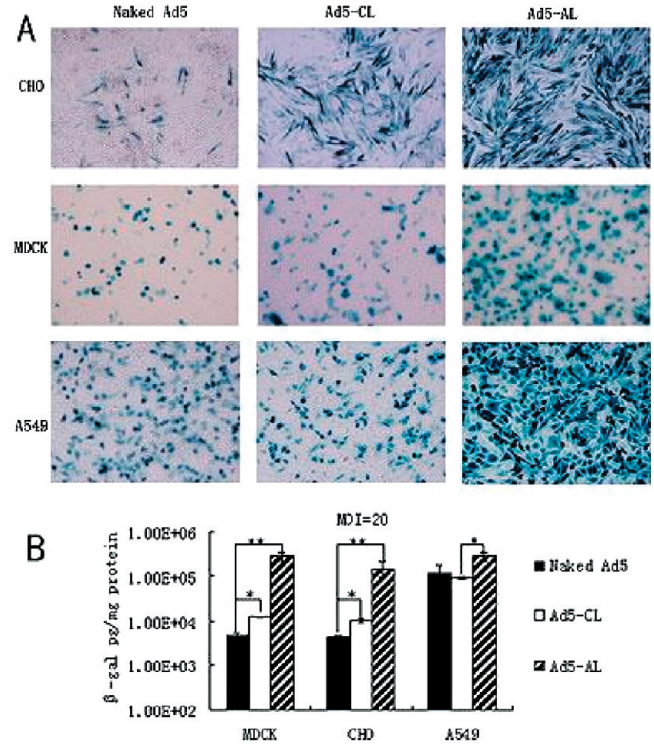

Figure 3. Gene transfer efficiency assay upon three cell lines. The cells were treated with 300 μL of three different formulations: the naked Ad5, Ad5 complexed with anionic liposomes (Ad5−AL) and Ad5 complexed with cationic liposomes (Ad5−CL) at MOI of 20 pfu/cell with a total lipid of 100 μg/mL. (A) Micrographs of CHO, MDCK and A549 cells treated with different formulations. After infection, cells were fixed and stained for β-galactosidase expression. (B) A quantitative detection of β-galactosidase gene expressions in MDCK, CHO and A549 cells. It was determined by using the β-galactosidase enzyme assay system. Data are mean ± standard deviation (SD) values ($n = 3$), $^*P < 0.01$, $^{**}P < 0.001$, ANOVA.

should be mentioned, PCS does not "measure" particle sizes. Rather, it detected the fluctuations of light signals caused by the Brownian motion of the particles to calculate their sizes. For the TEM sample preparation, the surface water was removed by air drying, which may cause the shrinkage of viral vectors, so that the mean diameter determined by TEM was smaller than that determined by PCS.

Compared to the corresponding blank liposomes, the particle sizes of Ad5−AL rose from 163 ± 12 nm to 211 ± 10 nm and the particle sizes of Ad5−CL increased from 186 ± 12 nm to 239 ± 15 nm. Meanwhile, the zeta potential of Ad5−AL rose from −48.8 ± 3.5 mV to −41.2 ± 2.2 mV and the zeta potential of Ad5−CL fell from 59.4 ± 3.8 mV to 48.9 ± 2.9 mV, compared with the blank liposomes. It was known the polydispersity index (PDI) could reflect the size distribution of nanoparticle population. As shown in Table 1, the PDI of Ad5−AL (0.268 ± 0.012) was smaller than that of Ad5−CL (0.330 ± 0.020), which indicated that the Ad5−AL had narrower size distribution than Ad5−CL did.

Cell Viability and Gene Transfer Efficiency. The result of the cell viability assay is shown in Figure 2. From Figure 2(A), it could be seen that no cytotoxicity of naked Ad5 was observed in A549 cells even at the highest concentration (MOI 80) as the cell viability (about 98%) remained roughly as same as it was seen in the nontransduced control cells (100%). At MOI 80, 40, and 20 pfu/cell, Ad5−CL showed decreased cell viabilities compared with Ad5−AL (84.5%, 84.1% and 85.3%, respectively). Figure 2(B) showed that there was no significant decrease in cellular viability when the cells were incubated with 50 μL of solution of Ad5−AL (MOI 20) at total lipids of 400, 200, 100 μg/mL, respectively. On the other hand, the cellular viability (48%, 61% and 85%) of A549 exposed to Ad5−CL decreased in a dose-dependent manner at the lipid concentrations mentioned above. These data demonstrated that the naked Ad5 and Ad5−AL were not cytotoxic to A549 cells, however, the Ad5−CL showed obvious cytotoxicity against A549 cells at total lipid concentrations of 400 μg/mL and 200 μg/mL.

Figure 3 describes the results of LacZ gene expression by either qualitative or quantitative detection in CAR overexpressing cells and CAR deficient cells. In our initial test,

the transduction efficiency mediated by Ad5−AL almost stayed at the same level when the total lipids ranged from 100 to 200 μg/mL (data not shown). Based on the transduction results and MTT assay results, the transduction reagents of both Ad5−AL and Ad5−CL were prepared at total lipids of 100 μg/mL (MOI 20).

From Figure 3 it could be seen that the gene expression detected by X-gal staining was consistent with the quantitative assay of β-galactosidase at a MOI of pfu/cell 20 and the LacZ gene expression mediated by naked Ad5 was poor in both CHO and MDCK cells for lack of CAR/integrin expression. In contrast, the LacZ expression of naked Ad5 was relatively higher in A549 cells, which was due to efficient CAR/integrin expression in A549 cells.[25,26] Formulating Ad5 with anionic liposome could significantly enhance the LacZ gene expression in all three cell lines, and the effect was more pronounced in the CAR/integrin deficient cell lines, CHO[27,28] and MDCK[29,30] cells. In the MDCK cells, the transduction efficiency of Ad5−CL increased over 2-fold to 12056 pg β-gal/mg protein compared with that of naked Ad5 (4681 pg/mg protein) and, moreover, Ad5−AL increased it 60-fold to 280168 pg/mg protein. In the CHO cells, the changing trend was similar to that observed in the MDCK cells. The naked Ad5 produced β-galactosidase almost at the same level between CHO and MDCK cells. Additionally, in Figure 3 (B), Ad5−AL showed higher gene transfer efficiency in MDCK cells than that in CHO cells, which indicated that the degree of enhancement mediated by Ad5−AL in CAR deficient cells might vary from cell to cell.

Ad5 Neutralization Measured by Transgene Inhibition. The presence of circulating antiadenovirus antibodies represents a special case of soluble factors in the serum that can affect virus trafficking and play an important role in the design and interpretation of clinical studies using adenovirus vectors.[31,32] Generally accepted replication inhibition assays as described in the literature[33−35] were performed with a wide array of assay parameters. In this study we took the transgene inhibition method.[36]

To assess if adenovirus in this complexed state was protected to any extent from neutralizing antibodies, antiserum raised against adenovirus was added to uncomplexed and liposome-complexed adenovirus preparations before infection, and the effect on resultant transduction efficiency was noted. Figure 4 described that the infectivity after preincubation with neutralizing serum was significantly reduced as compared with the expression measured in the absence of neutralizing antibodies. With uncomplexed adenovirus (naked Ad5), the neutralizing antiserum had a virtually complete inhibitory effect even at a dilution of 1:576. However, incubation with the neutralizing serum had a more limited effect on the infectivity of Ad5−AL and at the same inhibitory level the Ad5−AL required about 20-fold higher concentration of antiserum to block transduction and transgene expression effectively. As to Ad5−CL, the inhibitory trend was similar to the naked Ad5; the low transduction efficiency may be due to both the effects of serum inhibition and antibody neutralizing.[37]

Intracellular Distribution of Fluorescent Ad5. The fluorophore-conjugated Ad5 with a labeling degree of 90% was used to prepare the Ad5−AL and Ad5−CL through the methods mentioned above. Double color confocal laser

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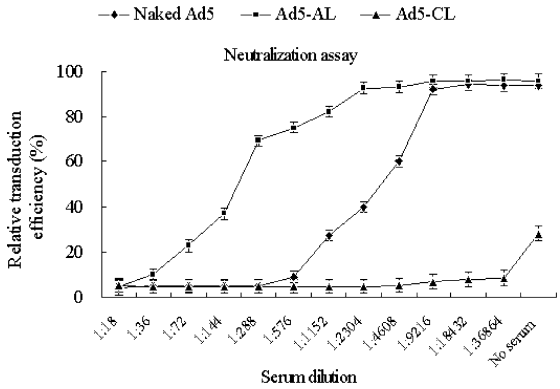

Figure 4. Neutralization determined by transgene expression inhibition. A549 cells were seeded in 96-well plates and infected with the naked Ad5, the formulation of Ad5 complexed with anionic liposomes (Ad5−AL) and the formulation of Ad5 complexed with cationic liposomes (Ad5−CL). Human serum was diluted ranging from 1/18 to 1/36864. Neutralization curves calculated from triplicate measurements are shown for each different formulation. The result was expressed as the relative transduction efficiency.

scanning microscopy (CLSM) was performed to determine the uptake mechanism of Ad5 vectors by A549 and MDCK cells and their intracellular location. As shown in Figure 5, the cellular uptake of naked Ad5 was much lower in CAR deficient MDCK cells (M1) than that in CAR overexpressing A549 cells (A1). Figure 5 (A2) and Figure 5 (M2) demonstrated that the uptake of Ad5 in both A549 and MDCK cells was significantly enhanced by the formulation of Ad5−AL and these two imagines also clearly showed that the red fluorescent Ad5 were found both in nucleus and extranucleus. Compared with naked Ad5, the Ad5−CL also facilitated the uptake of Ad5 in MDCK cells but the enhancement was much weaker than that observed in Ad5−AL infected cells. These results were consistent with the gene transduction results shown in Figure 3.

Discussion

Human adenoviruses are widely used as gene delivery systems, but adenovirus uptake is dependent on expression of Coxsackievirus-adenovirus receptor (CAR). Adenoviral vector 5 (Ad5) could not efficiently infect MDCK and CHO cells because they lack CAR expression.

Other studies have reported the use of cationic molecules to enhance viral uptake and subsequent transgene expression *in vitro* and *in vivo*.[38−41] In the present study, anionic liposomes were applied to encapsulate naked adenovirus in gene delivery system for the first time. To determine if this liposomal encapsulation allowed the adeno-vector to be transported into those CAR-negative cells and subsequently transduced, we infected CAR-positive (A549) and CAR-negative cells (CHO and MDCK) with Ad5−AL and compared the gene expression with naked Ad5 and Ad5−CL. The results showed that the enhanced gene expression mediated by Ad5−AL in CAR deficient cells was correlated with the enhanced Ad5 uptake. Ad5−AL exhibited much better transduction ability than Ad5−CL in all three cell lines ($P < 0.01$), which might be due to two reasons. First, according to the result of MTT assay, at the total lipid concentration of 100 μg/mL, the viability of A549 cells in the Ad5−AL group was better than that in the Ad5−CL group. The lower toxicity of the Ad5−AL group may be attributable to the higher gene expression in comparison to the Ad5−CL group. Second, one study made by McCray et al. demonstrated that one kind of Ca^{2+} chelator, the ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), caused a rapid, reversible drop in transepithelial resistance and facilitated gene transfer with retrovirus or adenovirus *in vitro*.[42] When Ca^{2+} chelator was applied to rabbit tracheal epithelia or human nasal epithelia *in vivo*, the transepithelial voltage decreased, and amiloride sensitivity was lost, suggesting that epithelial junctions opened. Other studies also indicated that Ca^{2+} chelator could enhance Ad5-mediated gene transfer to airway epithelial cells.[43,44] In our present study, the EDTA used in the Ad5−AL preparation is also one kind of Ca^{2+} chelator. Its structure and function are similar to EGTA. It might lead to enhanced Ad5 uptake and gene expression. Additionally, EDTA has been used in a phase I clinical trial to treat CF patients with *Pseudomonas* lung infections, and no toxic side effect was noted with the

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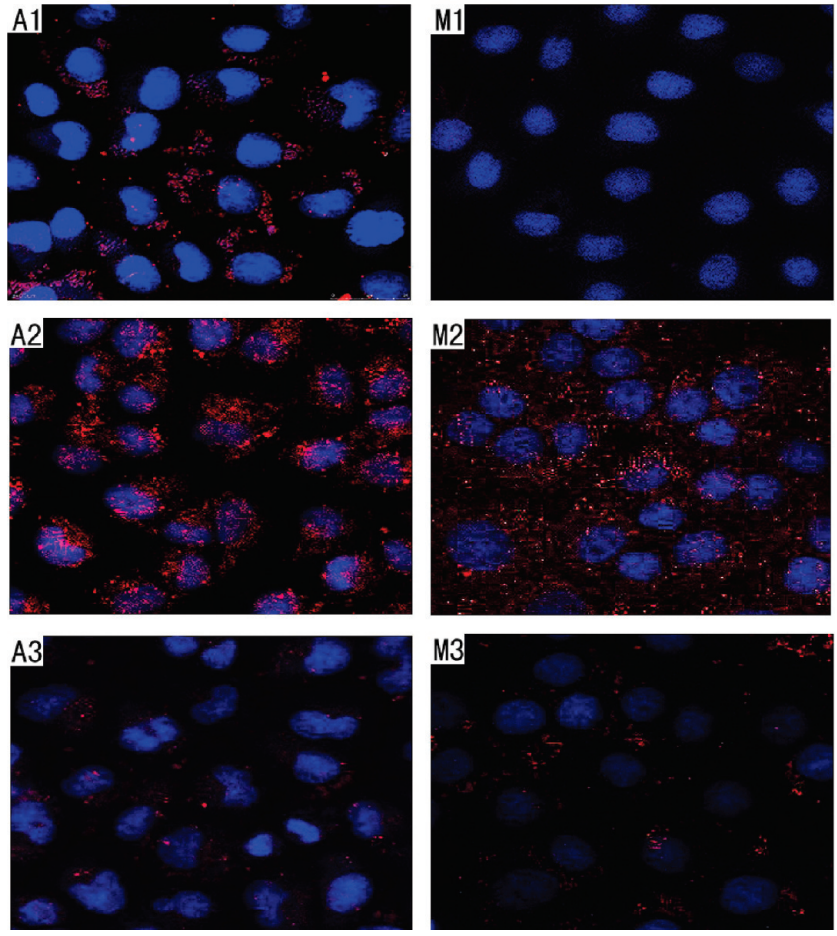

Figure 5. Intracellular distribution of red fluorescently labeled Ad5 following binding and internalization in A549 cells (A) and MDCK cells (M). In A1 and M1, the cells were infected with naked Ad5. In A2 and M2, the cells were infected with Ad5−AL, and in A3 and M3, the cells were infected with Ad5−CL.

administration protocol.[45−49] Therefore, EDTA in the formulation of Ad5−AL might be safe for its *in vivo* application. To further learn the role of EDTA, we also prepared the Ad5−CL and the naked Ad5 formulations exactly the same way the Ad5−AL were prepared. The results (Figures S(A)−S(C) in the Supporting Information) indicated that EDTA could enhance the transduction efficiencies of naked Ad5 in both A549 and MDCK cells whereas EDTA failed to improve the transfection efficiency of Ad5−CL in both cell lines. Moreover, it also demonstrated that the increased transduction efficiency was due not only to the presence of EDTA but also to the formulation factor of Ad5−AL as well as the properties of cell lines.

In the present study, a calcium-induced phase changes method was used to prepare the complex of Ad5 and anionic liposomes. In the literature of the calcium-induced phase change method,[50−55] phosphatidylserine (PS) is commonly used as an acidic phospholipid. In the current study, instead of PS, a more easily available chemical, CHEMS, acting similarly to a fatty acid to generate a negative membrane surface charge,[56] was first applied in calcium-induced phase change method for the preparation of anionic liposomes. As the aggregation or fusion events after Ca^{2+} addition proceeded, the turbidity decreased, which indicated that the sonicated liposomes changed from vesicle phase to precipitated phase. Subsequently, with the removal of Ca^{2+} by EDTA the lipids was redistributed and the viron particle was encapsulated into liposomes. Meanwhile the flocculent suspension became opalescent.

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One study[57] reported that the calcium-induced fusion of sonicated phosphatidylserine vesicles was of temperature dependence. The final average size as a function of the calcium incubation temperature was significantly increased with temperature increasing, peaked at the phase transition temperature, and then gradually decreased with the increasing of temperature, which indicated high temperature was beneficial to perform small particle size. The incubation temperature in the present study was chosen to be consistent with the physiological environment (37 °C), and the resulting particle size of Ad5−AL shown in Table 1 was the same as the data reported by Sun *et al.*[57] Additionally, during the preparation procedure, the virus did not undergo sonication and also was kept from contacting organic solvent, which reduced the risk of decreasing biological activity of virions. In fact, the transduction results of this study demonstrated that the encapsulated viruses were still biologically active after being complexed with anionic liposomes.

Earlier studies with adenovirus have shown that the mechanism of cellular uptake of adenovirus consisted of two distinct steps, initial attachment to the cells and subsequent binding of the viral penton base protein to cellular $a_v\beta_3$ and $a_v\beta_5$ integrins. The initial attachment to the cells occurs via an interaction between the fiber knob and several cellular receptors including the CAR[58,59] and the α_2 domain of major histocompatibility complex (MHC) class I molecules.[60] This initial binding allows close proximity with the cell and subsequent binding of the viral penton base protein to cellular integrins, promoting receptor-mediated endocytosis of the viral particle.[61,62] Therefore, the abundance of the CAR receptor, MHC class I receptors, and $a_v\beta_3/a_v\beta_5$ integrins on the surface of different cell types greatly influences the level of infection by adenoviral vectors.[63,64] Cells lacking CAR are more resistant to adenoviral infection and, consequently, they are poor targets for Ad5-associated tumor therapies.[65] This theory just gives interpretation to the uptake results mentioned above. For A549 cells, the CAR was overexpressing, which promoted the intracellular trafficking of Ad5 otherwise for the CAR deficient MDCK cells.

By combining adenovirus and liposomes, the advantageous characteristics of both viral and nonviral delivery systems can be exploited. For example, the liposomes have low immunogenicity, but meet significant barriers on endosomal escape and nuclear uptake. Adenovirus, however, possesses an inherent capacity to destabilize endosomes and enter nucleuses.[66] Therefore, a combination of liposomes and adenovirus might hold great potential for efficient *in vivo* gene delivery.

In summary, this present study showed that the complexes, Ad−AL, prepared by the calcium-induced phase change method could significantly enhance the gene transfer on CAR deficient cells mediated by Ad5 and also protected the adenoviruses from neutralizing by the human antiserum with antiadenovirus antibodies. Ad−AL exhibited significantly less cytotoxicity against A549 cells in comparison with Ad−CL and had a high ability to introduce the adenovirus into cytoplasm or nuclear accumulation. These findings suggested that anionic liposomes could significantly enhance the transduction ability

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of adenovirus in CAR deficient cells. The Ad−AL may be proved to be a useful strategy to delivery therapeutic genes into target cells.

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Supporting Information Available: Transfection efficiency comparison of naked Ad5, Ad5−AL and Ad5−CL, in which the Ad5−CL and the naked Ad5 formulations were prepared exactly the same way the Ad5−AL were prepared. This material is available free of charge via the Internet at http://pubs.acs.org.

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