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RESEARCH ARTICLE

Adenovirus or HA-2 fusogenic peptide-assisted lipofection increases cytoplasmic levels of plasmid in nondividing endothelium with little enhancement of transgene expression

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Abstract

Background Adenovirus-assisted lipofection has been reported to increase transfection efficiency through mechanisms potentially involving endosome escape and/or nuclear targeting activity. Similarly, transfection with the viral fusogenic peptide HA-2 of the influenza virus hemagglutinin can increase transfection efficiency. However, there are few studies examining the mechanism and intracellular trafficking of these viral and/or viral fusogenic peptide-assisted lipofections.

Methods and results Endosome escape was directly assayed with T7 RNA polymerase bound to plasmid (pTMβgal) expressing β-galactosidase under a T7 promoter to detect transcribable plasmid that escapes the endosomal compartment. Lipofection of pTMβgal with replication-deficient adenovirus (Ad5-null) at a multiplicity of infection (MOI) of 100 and 1000 increased cytoplasmic levels of transcribable plasmid by 24- and 117-fold, respectively, over lipofection alone, without an effect on total plasmid uptake. However, lipofection of pCMVβgal with Ad5-null at a MOI of 100 and 1000 increased transgene expression only seven- and eight-fold, respectively, over lipofection alone. Thus, a 24-fold increase in endosome escape saturated expression from pCMVβgal and provided only a seven-fold benefit in nondividing cells, which was not significantly increased with further increases in endosome escape. A cationic form of HA-2 (HA-K_4) also caused significant enhancements in endosome escape, as detected with the cytoplasmic transcription assay. However, HA-K_4 enhancement of endosome escape did not correlate with transgene expression from pCMVβgal, consistent with the detection of HA-K_4-mediated partitioning of plasmid to the insoluble fraction of the cell lysate.

Conclusion These results indicate that enhancement of endosome escape in nondividing cells does not fully alleviate rate limits related to nuclear import of the plasmid. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords endosome; hemagglutinin; gene therapy

Introduction

Adenovirus has the ability to transduce nondividing mammalian cells with high efficiency [1–5] due to efficient DNA packaging and protection, receptor targeting, endosome escape, microtubule transport to the nuclear pore, and

nuclear import. These features provide design strategies for nonviral gene transfer. The adenovirus fiber protein binds to the Coxsackie and adenovirus receptor (CAR) on target cells [6]. Internalization is mediated by five RGD sequences in the penton base that are bound by cellular integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [7]. The fiber is dissociated from the penton base early in the entry pathway [8]. About 80% of bound virus is internalized within 10 min. Following virus internalization, the penton base undergoes an acid-dependent conformational change and becomes hydrophobic to mediate endosome escape [9]. The virus in endosomes can escape to the cytoplasm within 5–15 min [8]. Agents such as chloroquine and monesin that inhibit endosome acidification block adenovirus penetration to the cytosol, but not virus internalization [10].

These remarkable activities of adenovirus have been used to enhance the delivery of plasmid to cells. Live adenovirus, replication-deficient adenovirus, or empty capsids augment the cell transfection of *unlinked* plasmid DNA [11–13]. Replication-deficient El^-E3^-Ad5CFTR or empty adenovirus capsid increases luciferase expression from pRSVluc in both dividing Cos-7 and HeLa, and this enhancement is abolished with chloroquine and anti-fiber antibody [11–13]. Hence, receptor-mediated endocytosis of adenovirus and the low pH of the endosome are both critical for the adenovirus-mediated enhancement of transfection [8,9,11–13].

Adenovirus has been linked to transferring–polylysine–DNA complexes and this has resulted in nearly 100% transfection of mouse hepatocytes, while unlinked adenovirus resulted in only 5–10% transfection [14]. Adenovirus or psoralen-inactivated adenovirus can also enhance the expression of unlinked 48 kbp cosmid DNA to levels approaching 100% transfection efficiency in HeLa [15]. These authors explained the enhancement to be the result of the endosome escape activity of the virus. However, a similar approach resulted in only 20% transfection efficiency with dividing human smooth muscle cells [16]. Similarly, mixing lipid–plasmid complexes with replication-deficient adenovirus yielded a moderate increase in transfection efficiency from a few percent to a level of 20–35% in subconfluent, dividing human or bovine endothelial cells [17,18]. These marked variations between linked and unlinked adenovirus and between hepatocytes (or HeLa) and smooth muscle cells (or endothelial cells) indicate that the mechanisms of adenovirus-assisted lipofection are not fully understood and that enhancement of endosome escape may not be sufficient for transgene expression in all situations. Directed measurement of adenovirus-mediated enhancement of endosome escape of plasmid has not been made.

Similarly, the fusogenic peptide derived from influenza virus has been employed for nonviral gene transfer. The virus binds cell surface sialic acid, is endocytosed, and escapes from the endosome with the help of hemagglutinin protein (HA). Endosome acidification causes a conformational change in HA, exposing the amphipathic fusogenic N-terminus of the HA-2 subunit which mediates endosome escape [19–21]. Fusogenic peptides increase transfection rates [22,23]; however, most of these observations were made in rapidly dividing cells, or were achieved in comparison to the polylysine DNA complexes. With HA-2-derived peptides, transfection efficiency for HeLa cells increased about two-fold in different liposome formulations [24]. Direct measurement of enhanced endosome escape of plasmid during HA-2-mediated gene transfer has not been made.

We sought to study these issues of mechanism (endosome escape vs. nuclear import) to identify critical rate limits during nonviral gene transfer to nondividing cells. In this work, we used a cytoplasmic transcription assay to quantify the cytoplasmic level of plasmid during adenovirus- and HA-2-assisted lipofections. Confluent endothelial cells were used at the time of transfection to evaluate the role of nuclear import as a rate-limiting step during nonviral gene transfer to nondividing cells to mitigate the complication of nuclear breakdown during mitosis.

Materials and methods

Cell culture

Bovine aortic endothelial cells (BAECs) (Cell Systems Corporation) were grown to confluence, passed at a high density (1 : 1 split) to 24-well culture plates, and then grown to dense confluence ($>100\,000$ per cm^2) for 2 days prior to lipofection. By flow cytometry with propidium iodide, 2-day post-confluent BAECs were measured to have less than 2% of the population in G2/M, demonstrating that BAECs were strongly contact-inhibited and growth-arrested at the time of their use for the lipofection experiments. Growth medium was Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated newborn calf serum, 0.30 mg/ml glutamine, 150 U/ml penicillin, and 0.15 mg/ml streptomycin (Gibco-BRL).

Transfection

Lipofectamine™ reagent (Gibco-BRL) was used in all transfections and weight ratios of 10 : 1 lipofectamine to pCMVβgal or pTMβgal and 5 : 1 lipofectamine to pCMVEGFP were used. For each transfection, 2.5 or 5 μl of lipofectamine™ (2 μg/μl) and 1 μg of plasmid were diluted into 50 μl of serum-free medium (OPTIMEM Reduced Serum Medium, Gibco-BRL) and incubated for 45 min. In some experiments, adenovirus (100 or 1000 MOI) or fusogenic peptides (10–50 μg) were added to the plasmid and incubated for 15 min prior to lipid addition. Then 150 μl of OPTIMEM medium was added to 50 ml of lipid/plasmid ($\pm$ adenovirus/HA) mixture, which was then placed on the cells (200 μl/2 cm^2 well). The cells were incubated for 2 h at 37°C, aspirated, fed 4% serum in OPTIMEM medium or DMEM with 5%

serum, and maintained for 2 days before assay. During the transfections, cells were frequently monitored for viability and adhesion.

Three types of adenoviral particles were used in this work: live adenovirus-2 (ATCC) produced in HeLa cells; Ad5-null, a replication-deficient adenovirus type 5 with E1 and E2 deleted (kind gift of Dr Thomas Shenk, Princeton University); and replication-deficient adenovirus-GFP (Ad-GFP, Quantum Biotechnology) with E1 and E2 replaced with the GFP gene. Since only a few lysines in the classical SV40 nuclear localization sequence are needed to condense plasmid, we designed and synthesized the N-terminal sequence of influenza hemagglutinin (HA) with a lysine tail (HA-K_4, GLFGAIAGFIENGWEGMIDGSSKKKK) (Sigma Genosys).

Plasmid fluorescence labeling and intracellular distribution

For fluorescence studies of plasmid internalization and localization, the plasmid pCMVβgal or pCMVEGFP was labeled with rhodamine using the Label IT reagent (Panvera). The plasmid endosomal localization was determined by co-localization of rhodamine-plasmid and fluorescent endosomal compartments labeled with 0.5 mM fluorescence dye FM 2-10 (Molecular Probes) [25]. After labeling cell membrane and rhodamine-plasmid lipofection, cells were imaged with a Zeiss Axiovert epifluorescent microscope at different time points. The plasmid nuclear localization assay was determined by the co-localization of rhodamine-plasmid and DAPI-stained nuclei. Total uptake of rhodamine-labeled plasmid (excitation/emission: 576/597 nm) was measured in intact cells (SLM-Amico fluorometer) and calibrated with known amounts of rhodamine-labeled plasmid. To evaluate the association of fluorescent plasmid with the insoluble fraction of the lysate, the cells were measured for rhodamine fluorescence after three cycles of freeze/thaw cycles, followed by centrifugation for 10 min at 400 *g*. The complete internalization of plasmid was confirmed by treating cells with DNase I (10 U, 10 min at 37°C) to remove plasmalemma-bound DNA before the assay.

Cytoplasmic transcription assay

The T7 RNA polymerase cytoplasmic transcription assay [26,27] was used to monitor transcribable plasmid in the cytoplasm after lipofection. The plasmid pTMβgal (1 μg) (kind gift of Dr Michael Welsh, Howard Hughes Medical Institute, Iowa City, IA, USA), which expresses β-galactosidase under the control of a T7 promoter, was incubated with 100 U of T7 RNA polymerase (Promega) in 50 μl of serum-free OPTIMEM medium. Pure lipofectamine (5 μl) was added to the pTMβgal/T7 RNA polymerase complex; the mixture was incubated for 45 min and then diluted to 200 μl with OPTIMEM medium. The cells were lipofected ($\pm$adenovirus or $\pm$ HA-K_4) for 2 h at 37°C. After 2 h, the mixture was aspirated; 4% serum in OPTIMEM medium was added; and the cells were maintained for different assays.

β-galactosidase assays

To measure percent transfection, cells were fixed with 1 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained with 1 mg/ml bromochloroindolylgalactoside (Xgal, Sigma-Aldrich), 5 mM K_3FeCN_6, 5 mM K_4FeCN_6, and 2 mM $MgCl_2$ for 4 h. The percentage of cells stained blue was counted in four random fields of view for each transfected monolayer and averaged. For total β-galactosidase expression, trypsinized cells were pelleted and resuspended at 10^7 cells/ml in incubation buffer containing 4% newborn calf serum in PBS with 10 mM Hepes (pH 7.2). The fluorogenic substrate fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes) was used following the manufacturer's directions. Total fluorescence (excitation/emission: 494/520 nm) was measured in an SLM-Amico fluorometer (Spectronic Instruments). Background enzyme activity and autofluorescence were subtracted using untransfected cells.

EGFP assay

Total fluorescence (excitation/emission: 488/507 nm) of EGFP from cell lysate supernatants was measured in the SLM-Amico fluorometer and calibrated with purified EGFP (Clontech). Cells isolated from each well were trypsinized, pelleted, resuspended in 100 μl of PBS, and lysed by three freeze–thaw cycles. Centrifugation for 10 min at 400 *g* removed insoluble debris.

Production of recombinant adenovirus particles

Adenovirus-2 particles were produced in 293 or HeLa cells. The 293 cells were grown to a density of 5×10^6 cells in T75 flasks, infected at a multiplicity of infection (MOI) up to 1000 for 90 min at 37°C, and then grown for 72 h. The cells were lysed by three freeze–thaw cycles and centrifuged at 200 *g*. Viral particles were purified by two rounds of CsCl gradients and one round of continuous CsCl gradient. CsCl was removed by dialysis against PBS containing 10% glycerol. The virus titer was determined by measurement of SDS-extracted viral DNA by a spectrophotometer, following the method of Challberg and Ketner [28] using the following formula: virus titer = A_{260} nm × (dilution factor) × (3.5×10^9 particles/ml), which is a measurement of intact assembled particles that serves as an estimate of plaque-forming units (pfu).

DNA binding and DNase protection assays

The binding capability of HA-K_4 peptide towards plasmid DNA was analyzed by agarose gel electrophoresis [29]. Purified pCMVEGFP (1 μg) was complexed with various

amounts of HA-K_4 peptide in PBS for 30 min at room temperature and subjected to 1% agarose gel electrophoresis. The DNase protection assays were conducted in the same condition, then 0.1 U (to see the protection effect) or 10 U (to match the post-transfection plasmid digestion assay of plasmalemma binding) of DNase I (Pharmacia) was added to the reaction mixture at 37°C for 10 min. Following the digestion, DNA was extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and separated on a 1% agarose gel.

Light scattering size determination

Particle size distributions of pCMVEGFP plasmid complexed with lipofectamine™ reagent and/or HA-K_4 were determined using dynamic light scattering (Brookhaven Instruments Corp.). The complexed particles were prepared by mixing plasmid and HA-K_4 (1 : 20, w : w); plasmid and lipofectamine (1 : 5, w : w); and plasmid, HA-K_4, and lipofectamine (1 : 20 : 5, w : w:w). Complexes of plasmid and HA-K_4 at ratio of 1 : 50 (w : w) were also tested and showed many different particles sizes which might have been caused by peptide aggregation (data not shown). A total of 1 μg of plasmid was used in the 200 μl reaction.

Results

Adenovirus enhances endosome escape, but not plasmid internalization

At 2 h after transfection, the FM 2-10-stained endosomal compartment and the rhodamine-plasmid displayed co-localization (Figures 1A–1C), indicating that the majority of plasmid resides in the endosomes at this time point. When confluent BAECs were lipofected with rhodamine-plasmid and visualized after 16 h by epifluorescence microscopy, the highly punctate staining in the cytoplasm of every cell demonstrated that the uptake and endosomal compartmentalization of plasmid were substantial (Figure 1D). In comparison, the nuclear level of plasmid was undetectable. When the same experiment was done in the presence of adenovirus, again there was no visible fluorescence inside the nucleus (data not shown). Adenovirus does not enhance the nuclear content of lipofected plasmid at levels detectable by epifluoroscence microscopy. Direct imaging cannot, however, be used to quantify the relative amounts of plasmid present in the endosomes or cytoplasm of these cells. In order to quantify the increase in cytoplasmic levels of plasmid in the presence of adenovirus, confluent BAECs were transfected with

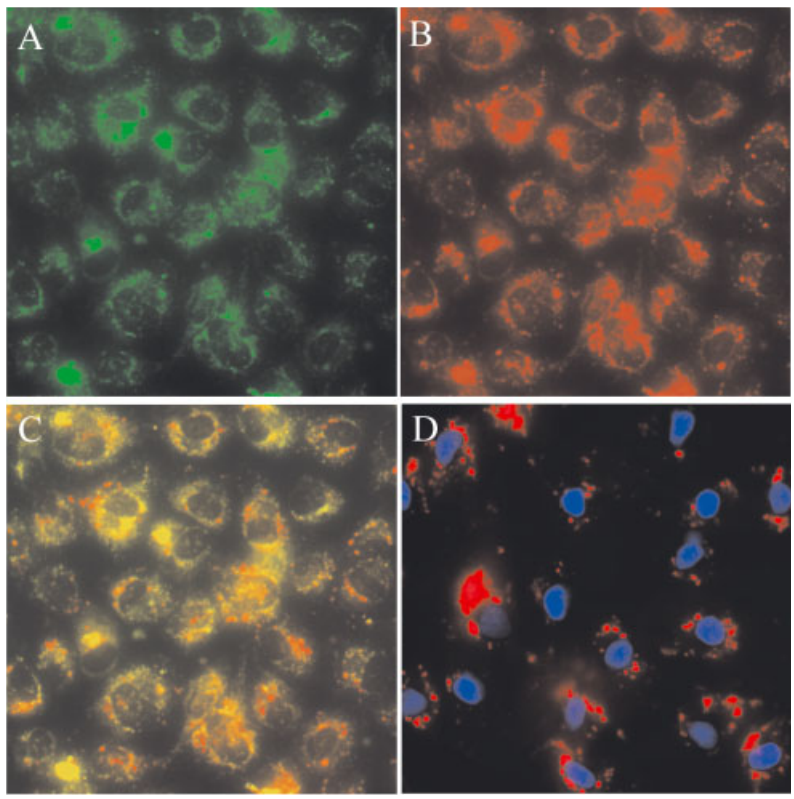

Figure 1. Lipofected fluorescent DNA was internalized by confluent endothelium and co-localized with internalized cell membrane, but was not localized in nuclei. Confluent BAECs were stained with fluorescence dye FM 2-10 (green) and then lipofected with 1 μg of rhodamine-pCMV*β*gal (red). The localization of fluorescence-labeled membrane and plasmid was visualized 1 h post-transfection. A shows the internalized cell membrane through endocytosis; B shows the internalized rhodamine-plasmid; and C is the overlay of A and B. D shows rhodamine-plasmid nuclear localization, which was visualized 16 h post-transfection. Cell nuclei were counter-stained with DAPI (blue)

plasmid pTMβgal (expressing β-galactosidase under the control of a T7 promoter) precomplexed with T7 RNA polymerase, cationic lipofectamine, and replication-deficient Ad5-null. The β-galactosidase activity was measured 12 h after transfection. As can be seen in Figure 2A, the addition of Ad5-null improved the cytoplasmic expression of β-galactosidase in a dose-dependent manner. At a MOI of 100, there was a 24-fold increase in the cytoplasmic expression of β-galactosidase compared with lipofection alone. At a MOI of 1000, there was a 117-fold increase in β-galactosidase expression. In separate cytoplasmic expression experiments, the addition of live adenovirus-2 viral particles (MOI $=500$) resulted in a 70-fold increase in the cytoplasmic expression of β-galactosidase at 10 h post-lipofection compared with matched controls that were lipofected without adenovirus-2.

In order to ascertain that the increase in endosome escape was not due to increased internalization of plasmid by the cells in the presence of adenovirus, confluent BAECs were lipofected with rhodamine-labeled pCMVβgal with or without Ad5-null and the total fluorescence intensity was measured. Addition of Ad5-null at a MOI of either 100 or 1000 did not increase the internalization of lipofected plasmid (Figure 2B).

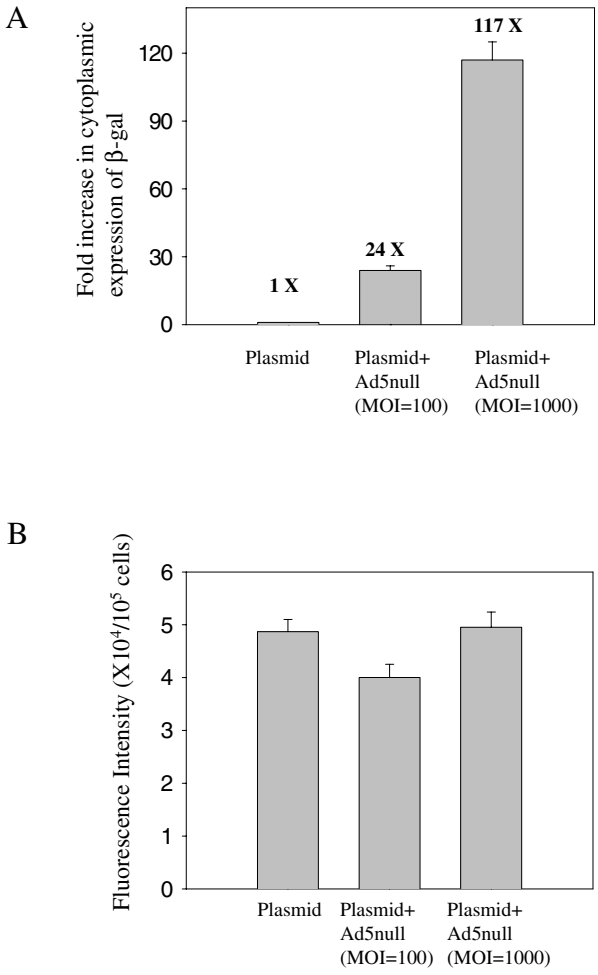

Figure 2. Adenovirus enhances endosome escape, as seen by the enhancement of the cytoplasmic expression of β-galactosidase after lipofectamine-mediated delivery of pTMβgal/T7 RNA polymerase (A), but it does not increase total fluorescent plasmid uptake (B)

Adenovirus transduction is not sufficient for transfection by unlinked pCMVβgal in confluent endothelium

To test whether adenovirus transduction can drive high efficiency lipofection in a nondividing cell population, confluent BAECs were lipofected for 2 h with pCMVβgal and replication-deficient Ad-GFP and then assayed for β-galactosidase activity and GFP fluorescence after 48 h. Transduction of confluent BAECs with Ad-GFP resulted in nearly 100% of cells expressing GFP after 48 h, but only about 10% of the cells expressing β-galactosidase from the CMV promoter as measured by Xgal staining (Figure 3), which is less than a four-fold increase compared with lipofection alone. Ad-GFP does not transport unlinked pCMVβgal into the nucleus with subsequently high levels of β-gal expression detectable by Xgal staining. The vacuoles seen in Figure 3A are a consequence of the high MOI needed to achieve high transduction.

The total expression of β-galactosidase from pCMVβgal was also measured fluorometrically (Figure 4). Despite the cytoplasmic levels of transcribable plasmid increasing from 24-fold to 117-fold above lipofection as the MOI increased from 100 to 1000, the expression of pCMVβgal was the same at a MOI of either 100 or 1000. Ad5-null, at a MOI of either 100 or 1000, resulted in a modest enhancement of transgene expression that was only seven- to eight-fold greater than lipofection alone (Figure 4). A 24-fold increase in the cytoplasmic level of plasmid was sufficient to saturate nuclear import of plasmid in nondividing BAECs. Further increases in endosome escape at a MOI of 1000 had no additional benefit in transgene expression from pCMVβgal.

HA-K_4 condensed and protected plasmid

We tested HA-2 fusogenic peptide as a tool to facilitate endosome escape. HA-K_4 formed tight complexes with plasmid, as seen in the gel-shift assay at >5 μg of HA-K_4 per μg of DNA (Figure 5A). At >5 μg of HA-K_4 per μg of DNA, HA-K_4 protected plasmid from nuclease digestion at a low concentration of DNase I (Figure 5B). With 0.1 U of DNase, plasmid was completely digested at 10 min at 1 or 2 μg of peptide per μg of DNA, but was completely protected at 20 μg of peptide per μg of DNA, incomplete protection being observed at 5–10 μg of HA-K_4 per μg of DNA. The DNA binding function of HA-K_4 was also monitored by dynamic light scattering. The complexes of lipofectamine and pCMVEGFP at 5 : 1 (w : w) ratio were 314 ± 46 nm in diameter. The average particle size was 461 ± 45 nm after adding lipid to HA-K_4/plasmid complexes, comparable to typical sizes of DNA–lipid particles of 100–500 nm [31].

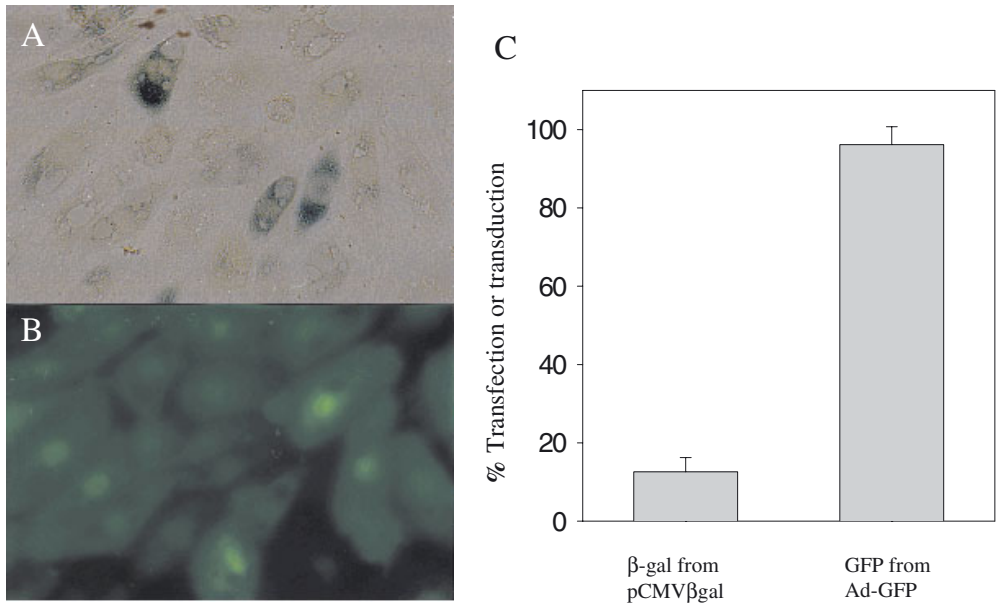

Figure 3. Transfection of confluent BAECs with adenovirus-GFP/lipofectamine-plasmid complexes. Confluent BAECs were lipofected with 1 μg of pCMV*β*gal/10 μl of lipofectamine in the presence of Ad-GFP (1000 MOI) and then assayed for *β*-galactosidase expressed from pCMV*β*gal (A) or GFP expressed from Ad-GFP (B) at 48 h after transfection. Despite transduction of nearly all cells by adenovirus, only a fraction of the cells expressed *β*-gal (C)

HA-K_4 increased plasmid internalization and endosome escape, but not transgene expression

Upon imaging of rhodamine-plasmid/HA-K_4 in lipofected cells, the plasmid displayed marked punctate cytoplasmic and perinuclear staining but no nuclear staining (not shown). However, when total cellular rhodamine-plasmid fluorescence in intact cells was measured, the HA-K_4-assisted lipofection had two-fold more signal than cells lipofected without HA-K_4 (Figure 6A). Complete internalization of HA-K_4 complexed plasmid was confirmed by a DNase treatment (10 U/ml for 10 min) of transfected cells, which reduced the fluorescent plasmid content of cells at 48 h post-transfection by less than 5% (data not shown). This confirmed that plasmid was taken into the cell and was not attached to the outer plasmalemma.

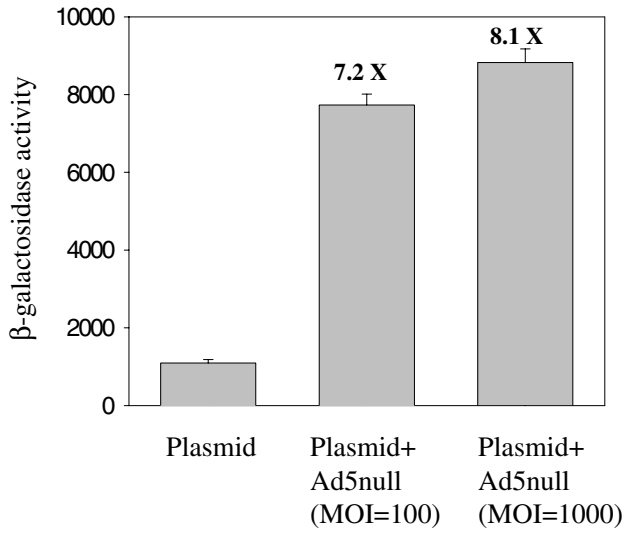

Figure 4. Lipofection of confluent BAECs with Ad5-null/lipofectamine-plasmid complexes. Confluent BAECs were lipofected with 1 μg of pCMV*β*gal and 10 μl of lipofectamine complexed with Ad5-null at a MOI of 100 or 1000. The cells were assayed for total *β*-galactosidase activity using the FDG assay after 48 h

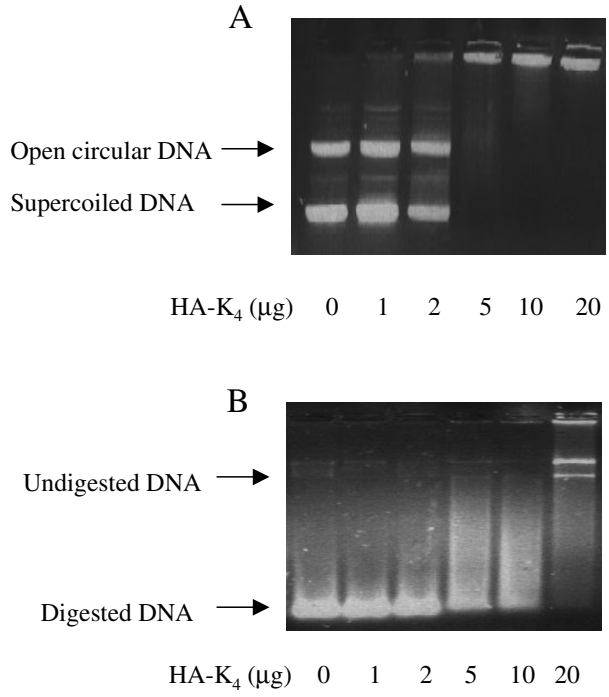

Figure 5. HA-K_4 peptide was able to bind and protect plasmid from a low concentration of DNase. A total of 1 μg of pEGFP was bound with different amounts of HA-K_4 and subjected to electrophoresis (A). HA-K_4 also protected plasmid from low-concentration DNase I (0.1 U) digestion (B)

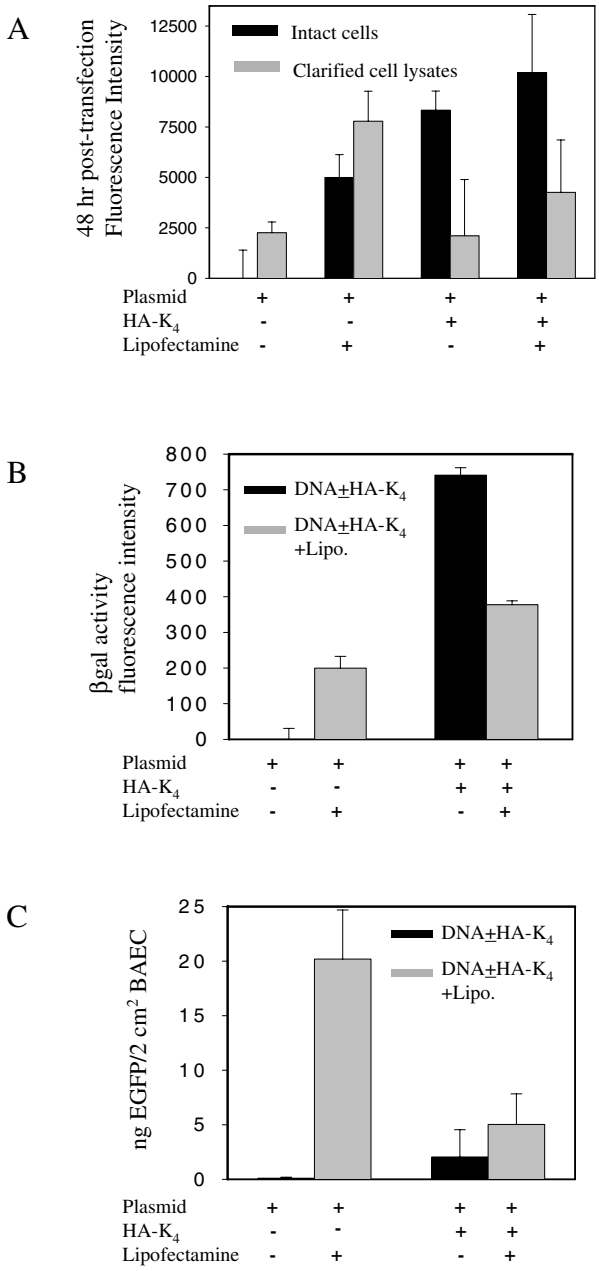

Figure 6. HA-K_4 enhanced internalization of fluorescent plasmid by confluent BAECs, but mediated association of plasmid with insoluble material inside the cell (A). HA-K_4 (50 μg) increased plasmid endosome escape in the cytoplasmic β-galactosidase transcription assay (B), which did not correlate with total transgene expression from pCMVEGFP when EGFP was used as the reporter (C). All transfections were lipofectamine-mediated (see details in the Materials and method section) unless noted otherwise

When cells were transfected with naked plasmid, no fluorescence was detectable in intact cells (Figure 6A). However, the post-centrifugation lysate (clarified supernatant) of these cells displayed fluorescence that had been quenched in the intact cells, presumably by packing or pH effects. It should be noted that the fluorescence of the soluble supernatant fraction is not a direct measure of plasmid that has escaped from the endosomes. As expected, lipofection of rhodamine-plasmid (no HA-K_4) markedly increased the amount of fluorescence in intact cells compared with delivering naked DNA. Again, post-centrifugation lysate of lipofected cells (no HA-K_4) displayed an increased fluorescence relative to intact cells. In contrast, HA-K_4/plasmid was readily internalized by cells in the absence of lipofectamine; however, the majority of this fluorescence was pelleted after centrifugation. HA-K_4 caused the plasmid to partition with the insoluble components of the intracellular milieu. Identical results were obtained when HA-K_4/plasmid complexes were lipofected. This binding of plasmid to insoluble components by HA-K_4 may be due to the hydrophobicity of the HA-2 sequence.

Consistent with the action of HA-2 as a fusogenic agent, the cytoplasmic transcription assay demonstrated that HA-K_4 increased endosome escape of plasmid (Figure 6B), as had been observed with Ad5null. The β-gal activity was very low in the pTMβgal/T7 RNApol transfection (no lipofectamine) and this activity could be increased by more than ten-fold when 50 μg of HA-K_4 per μg of plasmid was used. In separate *in vitro* transcription reactions, we observed that HA-K_4 reduced the incorporation of ^{32}P-UTP by T7 RNA polymerase into transcribed RNA (data not shown). Thus, the enhancement in endosome escape detected by the cytoplasmic transcription assay is a very conservative underestimate, since untranscribable plasmid may reside in the cytoplasm (potentially as the insoluble material detected in Figure 6A) that is not detectable by T7 RNA polymerase assay. HA-K_4 also increased by 1.9-fold the pool of transcribable DNA when plasmid was lipofected, but interestingly lipofectamine reduced the cytoplasmic expression of β-galactosidase from the pTMβgal/HA-K_4 complex (Figure 6B), while increasing uptake (Figure 6A).

Despite elevating by 1.9-fold the amount of transcribable plasmid in the cytoplasm after lipofection, complexation of 50 μg of HA-K_4 per μg of plasmid caused a marked reduction in the expression of marker protein from pCMVEGFP (Figure 6C). While the amount of transcribable plasmid in the cytoplasm after delivery with 50 μg of HA-K_4 was greater in the absence of lipofectamine than in the presence of lipofectamine (Figure 6B), the amount of expressed protein was greater in the presence of lipofectamine. In general, when HA-K_4 was added to plasmid prior to lipofection, transgene expression was markedly reduced.

Discussion

The overall efficiency of transfection is mainly dependent on the success of three steps: endocytosis, endosome escape, and nuclear localization [30–32]. Many laboratories have increased transgene expression with viral particles or viral peptides. However, the results may be cell-line- or cell-cycle-dependent [11–18]. To further understand the mechanisms that relate to adenovirus and viral peptide-assisted lipofection, we studied the subcellular plasmid trafficking using cytoplasmic and nuclear

transgene expression assays. We demonstrated that adenovirus could enhance by over two orders of magnitude the plasmid level in cytoplasm. Even with the 117-fold increase in cytoplasmic levels of transcribable plasmid caused by adenovirus, only about 10% of confluent BAECs expressed β-galactosidase from pCMVβgal with a consequent eight-fold increase in total protein expression despite viral transduction of all cells. The adenovirus had no capability to facilitate plasmid uptake (Figure 2B), which is expected since the viral particles were not linked to the plasmid. Adenovirus vectors can produce very high transduction efficiencies in endothelium both *in vivo* and *in vitro* [1–5]. We also found the same result in our adenovirus transduction assay (Figure 3B). In contrast to the poor nuclear import of plasmid, the adenovirus used efficient nuclear targeting to get its genome into the nucleus of nondividing cells, resulting in nearly 100% transduction as detected by EGFP expression (Figure 3B). These data indicate that even with marked enhancement of endosome escape due to the adenovirus, nuclear importation of plasmid is a major rate-limiting step that has to be overcome in order to achieve very high levels of expression or percent transfection by nonviral gene transfer. Adenovirus transduction is not sufficient for transfection by unlinked plasmid and the nuclear barrier to plasmid import is not altered by adenovirus.

HA peptide without a poly-lysine tail has been shown to increase the transfection efficiency of plasmids after transfection with liposome reagents [22,23]. No data have directly demonstrated that these peptides themselves can increase endosome escape of plasmid. We used the HA-2 peptide (no lysine tail) to transfect confluent BAECs with lipofectamine, but cell lysis resulted at a concentration of over 10 μg of HA with 2 cm^2 cells and 6 μg of lipofectamine (data not shown). The hydrophobicity and membrane fusion activity of HA-2 peptide even at neutral pH are the likely sources of this toxicity. With a poly-lysine tail, HA-K_4 was able to bind and protect plasmid from DNase digestion (Figure 5). This tight binding also decreased peptide toxicity towards cells at higher concentration. The HA-K_4/plasmid complexes, which are somewhat larger than material typically endocytosed, were able to enter the endothelial cells, as demonstrated by fluorescence microscopy and plasmid internalization assay (Figure 6).

The addition of the K_4 tail does not abolish the fusion activity of HA peptide in our study, a result consistent with others who used a large positively charged poly-lysine (with an average of 300 lysine residues) [22]. Nevertheless, our study is the first to measure directly the endosome escape of lipofected plasmid by adenovirus or fusogenic peptide HA-K_4 by use of the cytoplasmic transcription assays. In the HA-K_4-assisted lipofection, despite the more than ten-fold increase in plasmid internalization, the transgene expression level from pCMVEGFP was decreased. These data indicated that the plasmid that escaped from the endosome was unable to enter cell nuclei. Our plasmid distribution assay indicated that most of the plasmid that escaped from endosomes was bound with membranes and an insoluble fraction of cell organelles, likely due to the hydrophobic nature of HA-K_4 (Figure 6A). It is interesting to note that the penton base of adenovirus is lost from the capsid after endosome escape, which suggests a penalty for linkage of hydrophobic entities to plasmid in the cytoplasm. Efforts to enhance lipofection with hydrophobic sequences like those found in HA-2 may benefit from unpackaging mechanisms that release the hydrophobic peptide from the plasmid in analogy to the release of penton base by adenovirus.

In culture, uptake of plasmid is not rate-limiting (Figure 1). The cationic lipid–DNA complexes are internalized via endocytosis at a level of more than 10 000 plasmids per cell [33,34]. The neutral lipid DOPE undergoes phase transition from a bilayer phase at neutral pH to a hexagonal phase at low pH, which is believed to lead to disruption of the early endosome, albeit with low efficiency [35]. Treatment of cells with chloroquine, cytochalasin B, or ammonium chloride, all known endosome-disrupting agents, leads to an increase in transfection efficiency [36–38] in dividing cells, but with some toxicity associated with their use. Similarly, adding replication-deficient adenovirus to lipid–DNA complexes in transfecting subconfluent human or bovine endothelial cells can provide an increase in transfection efficiency from a few percent to a level of 20–35% [17,18,39]. We demonstrated that adenovirus caused large increases in endosome escape without a dramatic gain in transfection or expression in nondividing endothelium. Felgner and Ringold, using radioactively labeled complexes, showed that while 20–80% of the added polynucleotide was taken up by the cell, less than 1% of intact plasmid was found in the nuclear fraction [34]. Also, Capecchi observed that plasmid DNA injected directly into the nucleus of mouse LTK cells resulted in over 50–100% of the cells expressing thymidine kinase activity, compared with 0% cells when the plasmid was injected into the cytoplasm [40]. Zabner *et al.*, using vaccinia virus for cytoplasmic transcription of lipofected plasmids, showed that nearly 98% of COS-1 cells have plasmid in the cytoplasmic compartment, while only 10% express β-gal [35]. These results point to the fact that transport of plasmid across the nuclear pore can be a major rate-limiting step. In our earlier study, we found that attachment of nonclassical nuclear targeting sequences such as M9 sequences of the hnRNPA1 results in 80% transfection in confluent BAECs [30]. Such an approach to aid nuclear targeting may work synergistically with the endosome escape agent typified by HA-2, adenovirus, or cationic polymers. The cytoplasmic transcription assay provides a useful tool to evaluate inefficient mechanisms intermediate between uptake and nuclear-localized plasmid transcription.

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