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Characterization of gene delivery *in vitro* and *in vivo* by the arginine peptide system

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Abstract

We have reported previously that a basic peptide, arginine peptide, can be used as an efficient system for delivery of foreign genes. In this work, to better understand the mechanism of arginine peptide-mediated gene delivery, we further evaluated the process of cellular uptake and nuclear localization of the peptide/DNA complex. To investigate the effect of cellular proteoglycans on arginine peptide/DNA complexes, interactions between polyanionic glycosaminoglycans (GAGs) and peptide/DNA complexes were examined by the ethidium bromide interaction assay. Sulfated GAGs were found to relax the complexed DNA at low peptide/DNA charge ratios. Condensed peptide/DNA complexes facilitate cellular uptake, but their mechanism of uptake is poorly understood. Studies of various endocytosis inhibitors suggested that the peptide/DNA complex internalization involved the caveolar-related endocytosis pathway. A critical step in the gene delivery is the cytosol-to-nucleus transport of exogenous DNA following initial complex uptake. Nuclear localization of peptide/DNA complex was confirmed by confocal laser scanning microscopic observation. Further, we show that transfections with peptides result in an early accumulation of plasmid DNA in the nucleus of growth-arrested cells, which suggest nuclear transport. To assess the potential for arginine peptide as an agent for therapeutic gene delivery, *in vivo* complexed DNA transduction studies were performed. Mice were injected subcutaneously with the reporter gene β-galactosidase, resulting in high levels of gene expression in dermal tissue.

Keywords: Arginine peptide; Gene delivery; Nuclear localization; Proteoglycan; 293T cell

1. Introduction

In general, the plasma membrane of the eukaryotic cell is impermeable to the majority of proteins. However, several short regions of protein called protein transduction domains (PTDs) have been identified that are able to traverse the biological membrane. The most widely known PTDs are the human immunodeficiency virus 1 (HIV-1) transcriptional activator Tat protein (Fawell et al., 1994), the *Drosophila* homeotic transcription protein antennapedia (ANTP) (Derossi et al., 1994) and the herpes simplex virus structural protein VP22 (Phelan et al., 1998).

When PTD domains are covalently linked to full-length protein, or recombined to protein by in-frame gene fusion, they are able to deliver a protein, such as β-galactosidase, into the cell in active form (Schwarze et al., 1999). Sequence comparisons between the Tat, ANTP and VP22 PTD indicate the presence of basic amino acids, such as arginine or lysine, which may be important for contact with the negatively charged cell membrane. Arginine-only peptides were also able to translocate through cell membranes (Futaki et al., 2001). The transduction properties of arginine peptides are not limited to proteins, as they can deliver DNA as well. We recently demonstrated that short arginine peptides (R15), capable of forming complexes with DNA, promote efficient transfection in various mammalian cells types (Kim et al., 2003). Despite the high proficiency of arginine peptide as a carrier for such gene transfer, the molecular mechanism for this process is poorly understood.

Proteoglycans participate in a variety of functions ranging from formation of the extracellular matrix to cell proliferation and differentiation (Bernfield et al., 1999). Proteoglycans

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also mediate the cellular entry of many pathogens such as HIV-1, herpes simplex virus and *Neisseria gonorrhoeae* (Van Putten and Paul, 1995). Participation of the proteoglycans in the PTD internalization mechanism has been the subject of several investigations (Console et al., 2003; Fuchs and Raines, 2004; Goncalves et al., 2005). These molecules have been implicated in the first steps of the transfection process that begins with binding of the peptide/DNA complex to the cell membrane followed by cellular uptake (Kim et al., 2003). In addition, secreted proteoglycans can release the DNA from the cationic liposome/DNA complex (Belting and Petersson, 1997). Thus, despite only partial mechanistic understanding, the existing evidence suggests that proteoglycans are likely to play a key role in arginine peptide-mediated gene delivery.

Understanding the mode of entry for the peptide/DNA complex is crucial to developing efficient peptide-mediated gene delivery systems. A considerable body of data has accumulated, but the mechanism of internalization of cell-penetrating peptides is still controversial. Previous studies demonstrated that arginine peptides could be internalized even at low temperature (4 °C) (Futaki et al., 2001). Further investigations reported that internalization of these peptides is likely to be an energy-dependent and endocytotic process (Tomoki et al., 2002). Analytical microscopy techniques are widely used to study the peptide internalization mechanism. Recently, it was demonstrated that cell fixation results in the artifactual persistence of cell membrane-associated peptide, which could be misinterpreted as internalization—insofar as highly basic peptides have a strong affinity for the negatively charged cell surface and remain bound despite extensive washes (Lundberg and Johansson, 2002; Richard et al., 2003). Hence, such fixation artifacts may underlie a number of conflicting mechanistic studies, which justifies the re-evaluation of the molecular basis of peptide-mediated uptake. The internalization mechanism of the arginine peptide/DNA complex, for instance, would be distinguishable from that of peptide-only uptake, to the extent that the basicity shift caused by DNA binding is physically significant.

The efficiency of nuclear penetration of the complex is also vital to efficacious gene delivery. Large-molecule active nuclear transport is directed through the nuclear pore complex. This transport process is facilitated by a nuclear localization signal (NLS). NLS is a short sequence that has been identified as a cluster of four or more basic amino acids including arginine or lysine (Mattaj and Englmeier, 1998). Several studies have shown that Tat protein and VP22 protein can translocate through the plasma membrane and reach the nucleus (Dean et al., 2005). These observations suggested that arginine peptides might be able to deliver DNAs into the nucleus.

Although a number of gene delivery systems have shown promise as versatile cell culture transfection tools, there has generally been a substantial drop-off in transfection efficiency between the *in vitro* and the *in vivo* contexts, given the prohibitive physiologic sophistication of the latter case. For instance, the complete organism may present overwhelming numbers of negatively charged serum components that would nonspecifically interact with any peptide/DNA complexes.

In the present study, to determine the *in vivo* transfection efficiency of arginine-mediated gene delivery, complexes of the β-galactosidase encoding plasmid together with arginine peptides were injected into the mouse. Arginine peptides were successfully used to deliver β-galactosidase gene into mouse dermal tissue, demonstrating the potential for future therapeutic application.

2. Materials and methods

2.1. Materials

GenePORTER2 was obtained from Gene Therapy System (San Diego, USA). Lipofectin and β-gal assay kits were purchased from Invitrogen (Carlsbad, USA). Nystatin, filipin, chlorpromazine and cytochalasin B were obtained from Sigma–Aldrich (Saint Louis, USA). Luciferase assay kit purchased from Promega (Madison, USA). Arginine peptide (R15) was prepared by solid phase peptide synthesis using standard Fmoc (9-fluorenylmethyloxycarbonyl) chemistry. HPLC analysis indicated that the synthetic peptide was at least 95% purity. The peptide was dissolved in phosphate-buffered saline (PBS, pH 7.4) to appropriate concentrations. pcDNA3.1/His/lacZ, the expression vector for bacterial reporter gene *lac*Z under control of the human cytomegalovirus gene promoter, was purchased from Invitrogen (Carlsbad, USA). pCMV-Luc, containing the cDNA firefly luciferase reporter gene under control of human cytomegalovirus gene promoter, was constructed from pGL3-basic and pcDNA3.1/zeo.

2.2. Cell culture

The 293T cells were cultured in IMDM (Invitrogen, Carlsbad, USA) with 10% heat-inactivated fetal bovine serum and 1% antibiotics (streptomycin + penicillin). Cells were grown in 24-well plate and incubated at 37 °C incubator containing 5% CO_2 atmosphere.

2.3. Preparation of peptide/DNA complexes

Peptide/DNA complexes were formulated as described before (Kim et al., 2003). Plasmid DNA was diluted with 50 μl of PBS to a concentration of 10 μg/ml and peptide was dissolved in 250 μl of serum-free medium. To form the peptide/DNA complex, DNA solution was pipetted into the peptide solution and mixed vigorously by vortex. The complexes were incubated for 1 h at 25 °C, and 10% serum was added before use. GenePORTER2-mediated DNA formulations were produced as described by the manufacturer's protocol.

2.4. Ethidium bromide intercalation

DNA condensation was measured by quenching of ethidium bromide (EtBr) fluorescence as described earlier (Ruponen et al., 2002). Briefly, plasmid DNA (0.6 μg) was preincubated with EtBr either in arginine peptide or liposome (GenePORTER2) in 100 μl of PBS (pH 7.4). To examine the effects of GAGs,

glycosaminoglycans (25 μg/ml of chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, heparan sulfate and heparin) were added to the prepared peptide or liposome/DNA complexes. The GAGs and complexes were incubated for 1 h at 25 °C before assay. The fluorescence was measured using a Perkin Elmer LS 5B fluorescence plate reader (Perkin Elmer, Rodgau, Germany) at excitation wavelength 518 nm and emission wavelength 605 nm. Results are given as relative fluorescence and the value of 100% is attributed to the fluorescence of DNA with EtBr.

2.5. Peptide-mediated plasmid transfection

Since the maximal transfection efficiency was obtained at arginine peptide/DNA charge ratio 3.0 (Kim et al., 2003), unless otherwise noted transfection was performed at this charge ratio. Three hundred microliters of peptide/DNA complexes solution were gently added to the cells – which had been seeded into 24-well plate 18–24 h previously – and incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. Cells were then washed once with serum-free media and transferred to complete media for growth. After 48 h, β-gal gene expression was monitored. Transfection of growth-arrested cells was performed as described previously (Mortimer et al., 1999). Cells were incubated for 12 h with aphidicolin (25 μM, with FBS) before transfection. Transfection was performed in the presence of aphidicolin followed by further incubation of the cells for 24 h in aphidicolin-supplemented medium. Cells were then lysed by addition of 150 μl passive lysis buffer (Promega, Madison, USA) per well, and were assayed for luciferase activity.

2.6. Luciferase activity measurement

For the luciferase assay, cells (1×10^5 well^{-1}) were seeded in a 24-well culture plate at 18 h before transfection. The pCMV-Luc DNA (0.5 μg) was mixed with arginine peptide or liposome. After the transfection, cells were further cultured for 4–24 h and tested for luciferase gene expression. After the indicated time, cells were lysed by the addition of 150 μl passive lysis buffer per well. The cell lysate (100 μl) was then recovered for the luciferase activity assay. Light emission was measured by integration over 10 s at 25 °C using a luminometer (MicroLumat Plus LB96V, Berthhold, Germany). The total protein concentrations in cell lysates were determined using BCA assay (Pierce Chemical, Rockord, USA). Luciferase activity in each sample was normalized to the relative light unit (RLU) per milligram of cell lysate proteins.

2.7. β-Galactosidase assay

Expression of β-galactosidase genes was monitored after 48 h incubation at 37 °C in 5% CO_2 with β-gal assay kit according to the manufacturer's instruction. The transfected 293T cells were washed once with PBS and lysed with lysis buffer (20 μl/well). Cell debris was removed by centrifugation at 12,000 × *g* and 1 μl of the supernatant added to 50 μl of cleavage buffer containing β-mercaptoethanol and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) solution. After incubation for 30 min at 37 °C, the absorbance at 420 nm was measured. The protein concentration of each cell lysate was determined as stated above.

2.8. Effect of endocytosis inhibitors on transfection efficiency of peptides

Prior to incubation with peptide/DNA complexes, 293T cells were preincubated for 30 min either with chlorpromazine (10 μg/ml), filipin (2 μg/ml) and nystatin (50 μg/ml) at 37 °C or with cytochalasin B (52 μM) for 5 min at 4 °C as previously described (Nabi and Le, 2003; Richard et al., 2005). Alternatively, filipin was added during transfection. Then cells were incubated with peptide/DNA complex. At 2 h post transfection, the cultures were replaced with new complete media and further cultured for 48 h.

2.9. Cytotoxicity assay

The MTT assay was conducted essentially according to the manufacturer's protocol (Boehringer Mannheim, USA). Briefly, cells were plated on 96-microtiter plates in DMEM medium with 10% heat-inactivated fetal bovine serum in the presence of inhibitors. After 48 h incubation, MTT (0.5 mg/ml) was added to each well. Cells were incubated with MTT for 4 h. The insoluble formazan was dissolved overnight in solubilization solution. Cell viability was assessed by measuring the absorbance at 570 nm and expressed as the ratio of the A_{570} of cells treated with inhibitors over the control samples.

Viability of aphidicolin-treated cells was assessed by the evaluation of lactate dehydrogenase (LDH) release (Cytotoxicity Detection Kit; Roche) at 490 nm using an automatic microtiter plate reader.

2.10. Analysis of labeled peptide or liposome/DNA complexes in live cells by confocal microscope

To investigate the nuclear localization of peptide or liposome/DNA complexes in live cells, FITC tagging peptide (R15) and rhodamine-labeled DNA complexes or liposome/rhodamine-labeled DNA complexes were used. Labeled complexes were gently added to cells. To avoid possible experimental artifacts of cell fixation, at the end of incubation cells were washed three times with PBS, extensively washed again with PBS containing heparin (1 mg/ml) for complete removal of cell surface-bound materials as previously described (Kaplan et al., 2005). Distribution of fluorescently labeled complexes was visualized using a confocal scanning laser microscope LSM 5 (Carl Zeiss, Germany) equipped with a 40× objective, without fixing the cells.

2.11. In vivo administration

Hairless mice (body weight 25 ± 3 g) were used in this study. For subcutaneous administration, mice were injected under the skin with 300 μl PBS (divided and injected into three sites) containing 2.5 μg of plasmid DNA encoding β-galactosidase

and arginine peptide or liposome (GenePORTER2). Animals were sacrificed 96 h after injection and the skin was recovered. For β-galactosidase staining, the tissue was fixed for 18 h in a solution containing 2% formaldehyde and 0.2% glutaraldehyde, washed three times with PBS, and incubated for 24 h in staining solution [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 1 mg/ml)], 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$ in PBS at 25 °C. X-gal stained tissues were examined with a dissecting microscope at 8×.

3. Results

3.1. Effects of GAGs on arginine peptide/DNA complex

Previous reports suggested that secreted proteoglycans are inhibitory to cationic lipid-mediated DNA uptake and thereby gene expression (Belting and Petersson, 1997; Ruponen et al., 2002). This inhibition of the uptake of liposome/DNA complexes can be explained by way of proteoglycan-mediated dissociation of the complex. To evaluate the proteoglycan effect on arginine peptide/DNA complexes, arginine peptide/DNA complexes treated with soluble GAGs were studied using the EtBr intercalation assay. Condensation of DNA with cationic peptide results in displacement of EtBr from DNA causing a decrease in fluorescence signal. Negatively charged GAGs may disassociate cationic peptide from DNA or otherwise alter the conformation of complexes allowing binding of EtBr, thereby increasing the EtBr fluorescence (Ruponen et al., 2002).

DNA condensation and relaxation results are shown as a percentage of the fluorescence without peptide (i.e., DNA and EtBr). As shown in Fig. 1A, peptides formed tight complexes with the DNA at various arginine peptide(+)/DNA(−) charge ratios (1.0–30.0). Liposomes (GenePORTER2) formed loose complexes with DNA at low charge ratios (1.0–3.0), and formed tight complexes with DNA at high charge ratios (10.0–30.0). At the lower charge ratios (1.0–3.0), liposome formed loose association with DNA causing a 20% decrease in fluorescence, while arginine peptide formed tight complexes with DNA and caused a fluorescence decrease of 50%. These results indicate that arginine peptides condensed more effectively than the cationic liposomes at low charge ratios. The effect of GAGs on peptide/DNA complex was then tested. At low charge ratio (3.0), chondroitin sulfate B, heparan sulfate and heparin relaxed the peptide/DNA complex. Chondroitin sulfate A and C had less significant relaxing effect on the complexes (Fig. 1B). As expected, treatment with GAGs caused an increase in EtBr fluorescence from liposome/DNA complexes.

3.2. Internalization of the arginine peptide/DNA complex in the presence of endocytosis inhibitors

Earlier works showed that chloroquine, an inhibitor of the acidification of endosomal vesicles, addition enhanced the arginine peptide-mediated transfection (Kim et al., 2003). To further explore the possibility that peptide/DNA complex enters cells by an endocytosis mechanism, the effect of various endocytosis inhibitors on transfection efficiency was studied. Prior to the addition of peptide/DNA complex, the cells were preincubated in serum-free medium containing various endocytosis inhibitors, and then peptide/DNA complexes were added to the cells as previously described (Nabi and Le, 2003; Richard et al., 2005). As shown in Table 1, when cells were preincubated with the macropinocytosis inhibitor cytochalasin B, gene expression levels were moderately inhibited. The effect of chlorpromazine, an inhibitor of clathrin-dependent endocytosis, on arginine

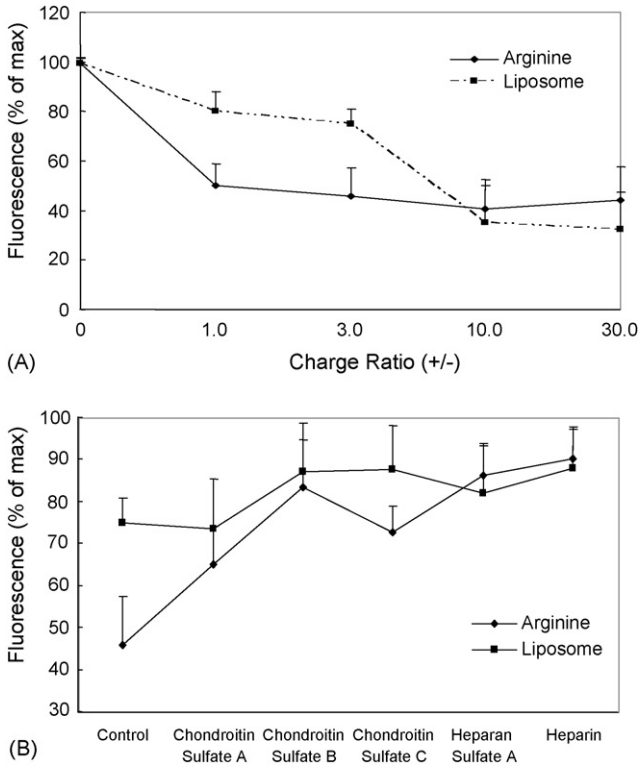

Fig. 1. Effects of glycosaminoglycans (GAGs) on arginine peptide/DNA complexes. (A) Effects of various charge ratios on condensing of plasmid DNA by arginine peptide and liposome (GenePORTER2); (B) Effects of GAGs on condensing of plasmid DNA by arginine peptide and liposome (charge ratio 3.0). The values are expressed as percentage of the EtBr fluorescence with DNA. Experiments were performed at least three times for each sample; data shown are mean value and S.D. for each measurement.

Table 1
Effects of endocytosis inhibitors on the arginine peptide-mediated transfection

Reagents	Expression percentage
Control	100 ± 7.15
Cytochalasin B (52 μM)	74.0 ± 6.91
Chlorpromazine (10 μg/ml)	77.2 ± 3.06
Filipin (2 μg/ml)	48.7 ± 5.14
Nystatin (50 μg/ml)	7.7 ± 0.85

Cells were pretreated with inhibitors at the given concentrations (Nabi and Le, 2003; Richard et al., 2005), and then peptide/DNA complexes were added to the cells. The cells were incubated for another 2 h in the presence or absence of inhibitors then changed with complete medium. The cells were harvested 48 h after transfection and performed β-gal assay. Experiments were performed at least three times at each sample; data shown are the means ± S.D. value of samples, described by the percentage of the expression of control cells without treatment of the inhibitors.

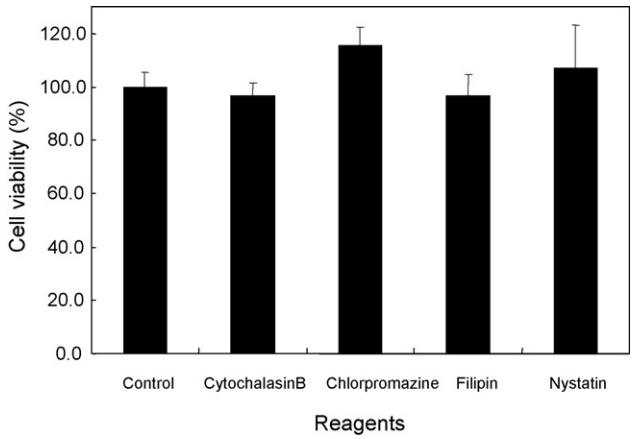

Fig. 2. The cytotoxicity of endocytosis inhibitors on the 293T cells. The 293T cells were exposed to various inhibitors at the given concentrations as described in Section 2. Cell cytotoxicity was determined by MTT assay. Experiments were performed at least three times for each sample; data shown are mean value and S.D. for each measurement.

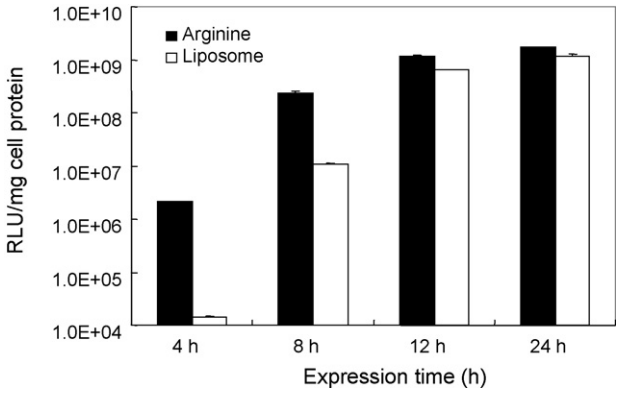

Fig. 3. Early transgene expression mediated by arginine peptide. The 293T cells were transfected with 0.5 μg pCMV-Luc complexed with arginine peptide or liposome (GenePORTER2). Transfections were stopped after 4, 8, 12 and 24 h. Luciferase activity was measured at indicated times. Experiments were performed at least three times for each sample; data shown are mean value and S.D. for each measurement.

peptide-mediated gene transfer was also examined. Treatment with 10 μg/ml chlorpromazine gave results similar to those seen with cytochalasin B. Filipin is a sterol-binding agent known to specifically block caveolae-mediated endocytosis (Richard et al., 2005). Treatment with this compound (2 μg/ml) resulted in a marked decrease in gene expression. Further indication of caveolae-mediated endocytosis of complexes was seen with nystatin, which alters the structure/function of glycolipid caveolae (Nabi and Le, 2003). Transfection efficiency was significantly inhibited by nystatin (50 μg/ml) treatment. These findings suggest that the internalization of peptide/DNA complexes may occur by a caveolae-dependent pathway. We observed no significant impairment to cell survival upon treatment with inhibitors: 293T cells displayed greater than 95% survival rate (Fig. 2).

3.3. Early transgene expression from arginine peptide-mediated transfection

Recently, it has been reported that cationic peptides, such as HIV–Tat and HSV–VP22, efficiently targeted exogenous DNA into the nucleus (Ritter et al., 2003; Dean et al., 2005). Accordingly, the proficiency of arginine peptide to deliver DNA to the nucleus was evaluated.

Due to earlier accumulation of plasmid DNA complexed with arginine peptide in the nucleus, the associated transcription – i.e., transgene expression – may be expected to outpace liposome agents that require mitosis for successful gene delivery (Mortimer et al., 1999). To test this hypothesis, cells were transfected with luciferase reporter gene complexed with arginine peptides, or with liposome (GenePORTER2). Transfection was stopped after 4, 8, 12 and 24 h and luciferase gene expression was immediately quantitated (Fig. 3). When transfection was stopped at 4 h, arginine peptide-mediated transfection showed higher gene expression – by at least 150-fold – than did liposome transfection. In contrast, transgene expression from liposomes increased significantly only at later time points.

3.4. Transfection of growth-arrested cells

To examine further the independence of peptide-mediated transfection from mitosis, we blocked the cell cycle with the anti-mitotic agent aphidicolin; that is, the peptide-based delivery should be efficient, but the liposome transfection should not, when mitosis is inhibited (Mortimer et al., 1999). Consequently, cells were treated with the aphidicolin and were then transfected with peptide/DNA complexes. Aphidicolin specifically arrests cells at the G1–S boundary without affecting processes involved in transfection (Ritter et al., 2003). As shown in Fig. 4, high transgene expression levels were observed from peptide-mediated transfections of either the aphidicolin-treated or untreated cells. However, the transfection efficiency of liposomes was sharply reduced in aphidicolin-treated (non-dividing) cells relative to control (dividing) cells.

Aphidicolin synchronization had no effects on 293T cells because cell viability and adhesion events of synchronized cells were not diminished, compared to untreated controls (data not shown).

3.5. Arginine peptide enhances nuclear localization of plasmid DNA

The delivery of exogenous DNA to the nucleus by arginine peptide was further confirmed by confocal microscopy. In view of the aforementioned interpretive difficulties associated with cell fixation (Lundberg and Johansson, 2002; Richard et al., 2003), we examined live cells under the confocal scanning laser microscope, forgoing any cell fixation. Accordingly, live cells were prepared as previously described (Kaplan et al., 2005), followed by the fluorescence imaging. Fig. 5 depicts a representative confocal image of live 293T cells treated with FITC-tagged arginine peptide and rhodamine-labeled DNA complexes at 37 °C for 4 h. Fluorescence signals were seen principally around the nuclear membrane and partly within the nucleus (arrows), indicating that successful delivery of plasmid DNA to the nucleus by arginine peptide.

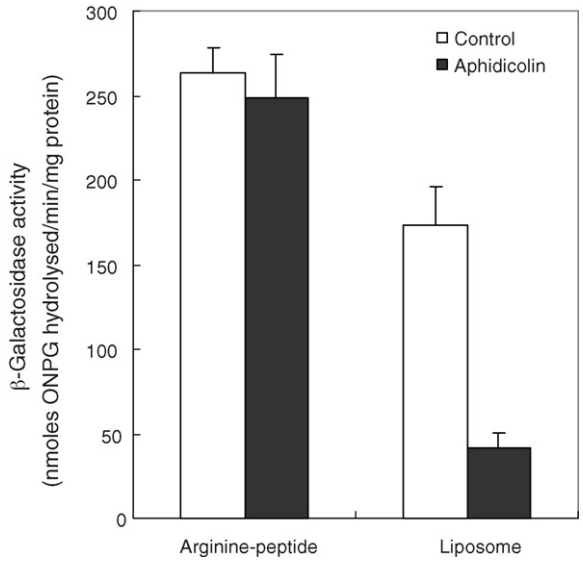

Fig. 4. Effect of aphidicolin on arginine peptide- and liposome-mediated transfection. To inhibit cell division, the 293T cells were incubated for 12 h in aphidicolin-supplemented medium before transfection, and then transfection was performed in the presence of aphidicolin (25 μM with FBS). Transfections were performed with 0.5 μg of pcDNA3.1/His/lacZ with arginine peptide (charge ratio 3.0) and liposome-mediated transfection was performed as described in the manufacturer's protocol. At 24 h post transfection, transfection efficiency of 293T cells was compared. Experiments were performed at least three times for each sample; data shown are mean value and S.D. for each measurement.

The delivery of exogenous DNA to the nucleus by liposome was also investigated. The 293T cells were incubated with rhodamine-labeled DNA/liposome complex or naked rhodamine-labeled DNA for 4 h. As shown in Fig. 6C, liposome/DNA complexes were predominantly detected in the cytoplasm, indicating complexes in entry into cells. However, naked DNA fluorescence was detected in extracellular surface of plasma membrane (Fig. 6F).

3.6. *In vivo* administration

The efficiency of arginine peptide-mediated gene delivery *in vivo* was examined by β-gal gene transfer to the tissue. Various charge ratios (0.5–30.0) of peptide/DNA complexes were subcutaneously injected directly into the of hairless mouse flank. Transgene expression was detected by X-gal staining of mouse skin tissue. As indicated in Fig. 7E, no staining for β-gal was found in the control groups. Staining was observed when pcDNA3.1/His/lacZ plasmid DNA was complexed with arginine peptides. This β-gal expression was dependent on the charge ratios of the peptide/DNA complexes (Fig. 7A–D). Maximum staining levels were found at a peptide/DNA charge ratio of 3.0 (Fig. 7B).

The *in vivo* transfection efficiency of peptides was compared to that of the commercially available cationic liposome reagent, GenePORTER2. As shown in Fig. 7B and F, arginine peptides yielded significantly higher transgene expression than the commercial transfection agent. The co-treatment of arginine peptide and GenePORTER2 with β-gal gene resulted in high levels of staining for β-galactosidase (Fig. 7G). Collectively, these data show that arginine peptides efficiently deliver the plasmid DNA *in vivo* and thus offer the potential of improved DNA transport for purposes of both *in vitro* gene delivery and *in vivo* therapeutics.

4. Discussion

The ideal peptide-mediated gene delivery system must satisfy a number of requirements. The bound, condensed peptide/DNA complex must be stable to the extracellular, as well as the intracellular environment. Once internalized, the complex must be impervious to degradative enzymes. Finally, the peptides must promote the nuclear translocation of plasmid DNA. The present work and a previously reported study (Kim et al., 2003) demonstrated that arginine peptides (R15) were proficient as *in vitro* and *in vivo* gene carriers.

Proteoglycans encompass a heterogeneous group of proteins that are highly polysulfated and thus negatively charged, as with the GAG polysaccharides. Although a number of studies suggest that proteoglycans act as receptors for cell entry of PTDs (Console et al., 2003; Fuchs and Raines, 2004; Goncalves et al., 2005), the literature is unsettled on whether proteoglycans are responsible for uptake of PTDs. Recent results have shown that binding of heparan sulfate is necessary for oligoarginine internalization (Fuchs and Raines, 2004; Goncalves et al., 2005). However, this constraint does not apply to conju-

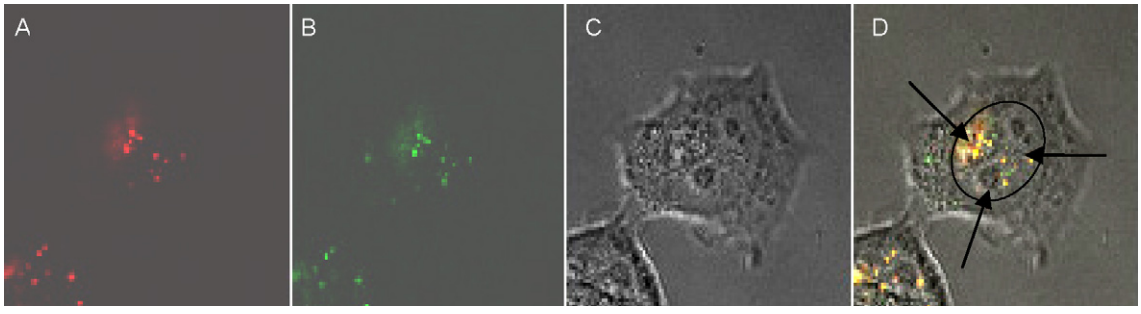

Fig. 5. Nuclear localization of arginine peptide/DNA complexes in live cells. The 293T cells were treated with FITC-labeled arginine peptide/rhodamine-labeled DNA complexes. The cells were harvested 4 h after transfection and washed as described in Section 2 to remove surface-bound complexes. And then the samples were examined by confocal laser scanning microscope without fixation. (A) Rhodamine-labeled DNA (red); (B) FITC-labeled peptide (green); (C) differential interference contrast (DIC); (D) merge (yellow fluorescence indicates colocalization of peptide and DNA). The circle is drawn around the nucleus.

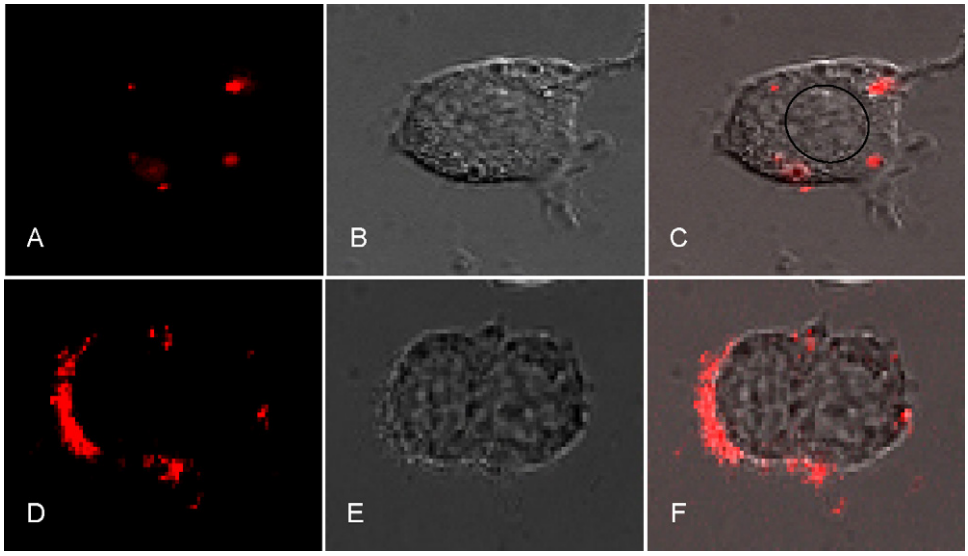

Fig. 6. Cellular localization of liposome/DNA complexes in live cells. The 293T cells were treated with rhodamine-labeled DNA/liposome complexes. The cells were harvested 4 h after transfection and washed as described in Section 2 to remove surface-bound complexes. And then the samples were examined by confocal laser scanning microscope. (A) Rhodamine-labeled DNA (red)/liposome complex; (B) differential interference contrast (DIC); (C) merge; (D) naked rhodamine-labeled DNA (red); (E) differential interference contrast (DIC); (F) merge. The circle is drawn around the nucleus.

gated TAT internalization (Silhol et al., 2002; Violini et al., 2002).

In light of previous studies reporting the reduction of arginine peptide-mediated gene expression upon treatment with exogenous GAGs (Kim et al., 2003), the present report examines the effect of GAGs on the arginine peptide/DNA complex. Our results confirm that GAG-induced relaxation of complexed DNA depends on the particular type of GAG. These data indicate that GAGs interact with arginine peptide/DNA complexes and may alter their transfection properties. Similar results were observed with cationic lipid/DNA complexes (Belting and Petersson, 1997) and cationic polymer/DNA complexes (Ruponen et al., 2002). Combining all these results suggest that proteoglycans may have a dual role in modulating arginine peptide-based gene delivery. First, cell surface proteoglycans mediate the binding of peptide/DNA complexes to the cell, i.e., they may act as receptors for peptide/DNA complex entry (Kim et al., 2003). Second, extracellular proteoglycans may affect peptide-mediated transport efficiency by directly influencing the peptide–DNA interaction itself. In this context, for successful gene delivery, high peptide/DNA charge ratios would be essential to out-competing the negatively charged proteogly-

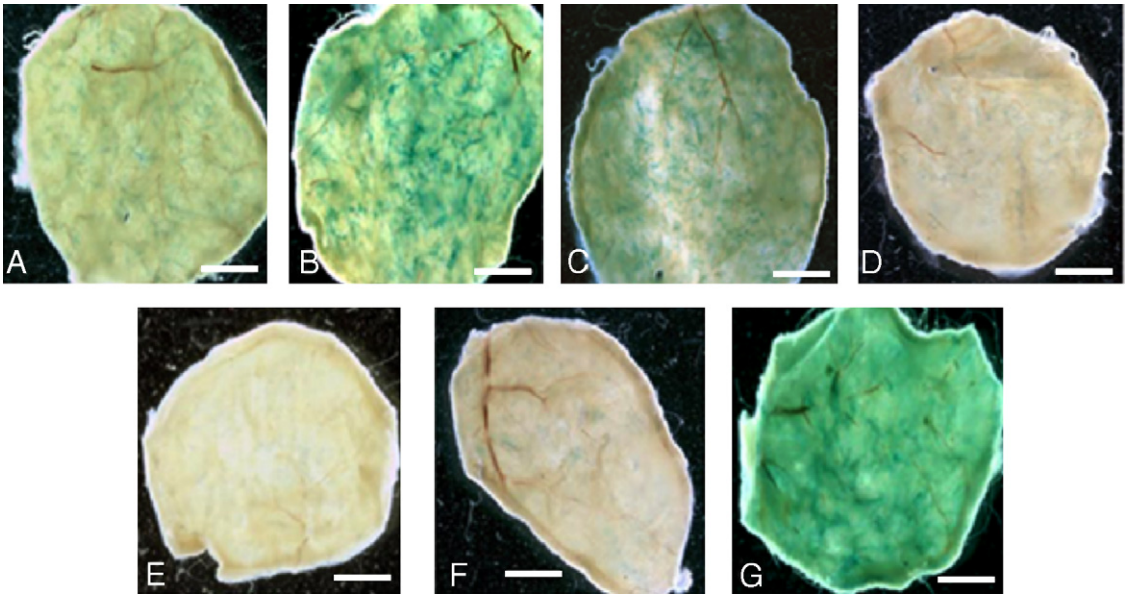

Fig. 7. X-gal staining of mouse dermal tissue at 96 h after subcutaneous injection of arginine peptide/DNA complexes. DNA encoding β-galactosidase delivery and skin tissue staining were performed as described in Section 2, and examined with a dissecting microscope. The skin of mouse injected with arginine peptide/DNA complexes, at charge ratio 0.5 (A); charge ratio 3.0 (B); charge ratio 15.0 (C); charge ratio 30.0 (D). The skin of control mouse injected with naked DNA (E); liposome with DNA (F); co-treatment of arginine peptide and liposome (G). Scale bar = 1 mm.

can. Recently, Goncalves et al. (2005) reported that nonaarginine (R9) has a strong affinity for heparin sulfate (binding constant: $3.1 \times 10^6\ M^{-1}$), underscoring the significance of complex binding strength and charge ratio as considerations in establishing the arginine-mediated gene delivery system.

Despite the general acceptance of PTDs as cellular delivery vectors, the mechanism by which these peptides translocate across the membranes has remained controversial. Moreover, the available quantitative studies mainly concern PTDs alone, whereas only a few reports have dealt with cargo molecules/peptide combinations. With the field of peptide-based DNA transport still in its infancy, establishing the molecular mechanisms for peptide/DNA delivery will become vital to eventually developing safe and effective clinical applications.

Previously, we suggested the involvement of an endocytosis mechanism on arginine peptide-mediated gene delivery (Kim et al., 2003). In the present work, we gained further mechanistic insight by examining the influence of endocytosis inhibitors on internalization of peptide/DNA complexes. The possible involvement of caveolae in internalization of complexes was studied. Both filipin and nystatin are known to disrupt caveolae formation and inhibit caveolae-mediated internalization (Couet et al., 2001; Ruponen et al., 2002). Upon treatment with these compounds, significant inhibition of transfection was observed, suggesting that arginine peptide-mediated gene transfer is at least partly by caveolae-dependent endocytosis. The recent literature shows that cellular uptake of PTDs is not restricted to a single type of endocytosis (Fittipaldi et al., 2003; Wadia et al., 2004; Deshayes et al., 2005). Richard et al. (2005) proposed that there may be differences in the respective uptake mechanisms for PTDs versus PTD/cargo molecule complexes. In the case of free PTDs, the cluster of basic amino acids is likely to be fully exposed to cellular molecules. In contrast, for PTDs bound to cargo molecules such as DNA, sequestration of the basic amino acids may have been a factor in the size of the complexes. Interestingly, recent studies have proposed that the size of the complex plays a crucial role in the internalization (Simeoni et al., 2003; Rejman et al., 2004).

Recently, Ritter et al. (2003) suggested that NLSV 404, a peptide vector with many positively charged amino acids, successfully delivered the DNA into nucleus. Since the nuclear membrane is the major barrier to the nonviral vector, understanding the mechanism of arginine-based nuclear translocation of DNA would be expected to accelerate the development of efficacious gene delivery tools. Our results showed that arginine peptide could transport fluorescently labeled DNA into the nuclei of 293T cells. In view of the confusion in the literature over the PTD distribution in fixed cells (Richard et al., 2003; Thoren et al., 2003), we examined the nuclear localization of peptide/DNA complex in live cells. As shown in Fig. 5, arginine peptide successfully delivered plasmid DNA into the nucleus. Notably, as indicated in Fig. 5D, colocalization of red (DNA) and green (peptide) fluorescence was detected in the nucleus, indicating that DNA and peptide were still complexed. This result compares well with previous PEI-mediated (Godbey et al., 1999) and polylysine-mediated (Kilink et al., 2001) gene delivery studies, which have found the localization of complexes in the nucleus. Similarly, it was also reported that arginine-rich protamine, when condensed with DNA, was able to translocate through the nuclear membrane in condensed form (Masuda et al., 2005). In the nucleus, protamine regulates the intra-nuclear localization towards the nuclear matrix-rich regions, where it plays an important role in gene translation (Martin et al., 2004; Masuda et al., 2005).

Next, the time course for transgene expression using arginine peptide and liposome vectors was evaluated. Mortimer et al. (1999) reported that plasmid DNA/liposome complex accumulated in the nucleus in a mitosis-dependent manner, requiring that some time elapse for development of transgene expression. In contrast, a peptide agent with the property of transporting DNA rapidly to the nucleus, regardless of mitosis, would be expected to express the reporter gene much earlier than the liposome-based system. Our present results (Fig. 3) showed such early gene expression; in particular, experiments with non-dividing cells further indicated the involvement of a nuclear translocation mechanism (Fig. 4). Our data support the view that arginine peptide efficiently delivers plasmid DNA from the extracellular milieu to the intracellular nuclear compartment.

Significantly, we were able to extend our studies to the *in vivo* model. It is well known that *in vitro* transfection is typically far more efficient than the corresponding *in vivo* gene delivery, since the whole organism imposes the extracellular matrix, inhibitory biological fluids and myriad other supramolecular barriers (Brown et al., 2001). The highest *in vivo* transfection efficiency observed for a peptide/DNA complex was at a molar charge ratio of 3.0. The same result was obtained for *in vitro* transfection in the presence of serum (Kim et al., 2003). However, in our EtBr interaction assay, some GAGs caused the release of DNA from complexes at the charge ratio of 3.0. These data suggest that additional factors help govern the efficiency of gene transfer. For example, overly strong peptide–DNA interaction may prevent the spatially appropriate release of DNA to the nucleus (Lucas et al., 2005). These results imply that optimizing the charge ratio will be important to an efficient arginine-based gene delivery system. Thus, we conclude that a charge ratio of 3.0 is suitable for achieving the required compactness in the complexed DNA and the sustained diffusion in the dermal tissue.

A small excess of positive charge was also found necessary for cationic liposome/DNA complexes to efficiently transfect *in vivo* (Song and Liu, 1998). This excess positive charge probably binds with cell membranes or negatively charged molecules in the tissue fluids. It may be that the modicum of excess positive charge protects from degradation of peptide/DNA complexes in such enzymatically hostile environments, which is consistent with the study of Rittner et al. (2002). In their *in vivo* transfections, basic peptide ppTG1 yielded the highest luciferase activity at low peptide/DNA complex charge ratios (1.8–2.1). Similar results were obtained from the polyethylenimine (PEI)-based gene delivery. High levels of luciferase expression were found when DNA was complexed with PEI at a ratio of 4 (N/P ratio) (Goula et al., 1998).

It is also noteworthy that the co-treatment of peptide with liposome induced significant increases in transfection, raising the possibility that peptides co-complexed in solution with lipo-

some might be useful for elevating transfection levels in gene therapy *in vivo*.

In conclusion, the results of our present and previous works (Kim et al., 2003) demonstrate that arginine peptides hold promise as vectors to efficiently transport plasmid DNA *in vitro* and *in vivo*.

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