Enhancement of Gene Delivery Using Novel Homodimeric Tat Peptide Formed by Disulfide Bond

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Cationic liposomes have been actively used as gene delivery vehicle because of their minimal toxicity, but their relatively low efficiency of gene delivery is the major disadvantage of these vectors. Recently, cysteine residue incorporation to HIV-1 Tat peptide increased liposome-mediated transfection compared with unmodified Tat peptide. Therefore, we designed a novel modified Tat peptide having a homodimeric (Tat-CTHD, Tat-NTHD) and closed structure (cyclic Tat) simply by using the disulfide bond between cysteines to develop a more efficient and safe nonviral gene delivery system. The mixing of Tat-CTHD and Tat-NTHD with DNA before mixing with lipofectamine increased the transfection efficiency compared with unmodified Tat peptide and lipofectamine only in MCF-7 breast cancer cells and rat vascular smooth muscle cells. However, cyclic Tat did not show any improvement in the transfection efficiency. In the gel retardation assay, Tat-CTHD and Tat-NTHD showed more strong binding with DNA than unmodified Tat and cyclic Tat peptide. This enhancement was only shown when Tat-CTHD and Tat-NTHD were mixed with DNA before mixing with lipofectamine. The effects of Tat-CTHD and Tat-NTHD were also valid in the experiment using DOTAP and DMRIE instead of lipofectamine. We could not find any significant cytotoxicity in the working concentration and more usage of these peptides. In conclusion, we have designed a novel transfection-enhancing peptide by easy homodimerization of Tat peptide, and the simple mix of these novel peptides with DNA increased the gene transfer of cationic lipids more efficiently with no additional cytotoxicity.

Keywords: Tat peptide, gene therapy, liposome, transfection

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liposome-mediated transfection in cell cultures and in vivo upon local injection [9, 21, 28]. Others, however, showed that the addition of cysteine is needed for the sufficient transfection of DNA [15, 19]. Addition of terminal cysteines to lysine-rich peptides condensed DNA stably [18], and disulfide bond formation was also shown to increase the stability of DNA–peptide interaction [17, 22]. Moreover, cysteine residue incorporation to Tat peptide increased liposome-mediated transfection more efficiently than unmodified Tat peptide [19, 32]. However, there was no report on homodimeric or cyclic Tat peptide using the disulfide bond. Therefore, we designed a novel modified Tat peptide having homodimeric and closed structure simply by using the disulfide bond between cysteines to develop a more efficient and safe nonviral gene delivery system. To this end, we evaluated the potency of these peptides in the MCF-7 breast cancer cell line and primary cultured rat vascular smooth muscle cells by luciferase and GFP gene expressions, and measured the mechanical interaction between these peptides and DNA using gel retardation assay.

**MATERIALS AND METHODS**

**Cell Line and Cell Culture**

Human breast cancer cell line MCF-7 was obtained from the Korean Cell Line Bank. The aortic vascular smooth muscle cells (VSMC) isolated from 11-week-old male Sprague–Dawley rats were generously provided by Hyoung Chul Choi (Department of Pharmacology, School of Medicine, Yeungnam University, Korea). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained in a humidified incubator-

**Peptide Synthesis and Modification**

The peptide used in this study was derived from the Tat protein of HIV-1. The Tat peptide corresponds to the nuclear localization signal (NLS) sequence of 12 amino acids (49–60; RKKRRQRRRPPQ). The peptide was modified at either the N- or C-terminus by the addition of cysteine (Table 1). All of the peptides described in this study were synthesized by Peptron, Inc. (Korea). Briefly, peptides were synthesized by the Fmoc-SPPS solid phase peptide synthesis method using APS-48S (Peptron Inc., Korea) and purified by reverse-phase HPLC using a Vydac Everest C18 column (250 mm × 22 mm, 10 µm). Elution was carried out with a water–acetonitrile linear gradient [3–40% (v/v) of acetonitrile] containing 0.1% (v/v) trifluoroacetic acid. For the formation of homodimeric peptides, each monomeric peptide was dissolved in 0.1 M ammonium bicarbonate in acetonitrile:water (v:v=1:1) (1 mg/ml). The reaction mixture was stirred at atmosphere until the reaction was complete. The reaction was monitored by Ellman test [3] and LC/MS. The mixture was lyophilized and then purified by reverse-phase HPLC. The average homodimeric conjugation yield was over 80% by HPLC monitoring of completed reaction samples (data not shown). The molecular weights of the purified peptide were confirmed using LC/MS (Agilent HP1100 series, USA). Purified peptides were resuspended at a concentration of 10 µg/µl in distilled water and kept at −70°C prior to further use.

**Formation of Complex and Transfection**

The pcDNA-Luc containing the firefly luciferase reporter gene and the plasmid coding for green fluorescent protein (pCMVtNT-GFP) were obtained from Welgene (Korea). DNA plasmids were amplified in the *Escherichia coli* XL1-Blue strain and purified by a maxi-kit (Qiagen Inc., USA) according to the manufacturer’s instructions. DNA purity was determined by agarose gel electrophoresis and by measuring the optical density (OD). DNA having OD260/OD280≥1.8 was used in this study. The pcDNA-Luc and pCMVtNT-GFP were used at a concentration of 0.3 µg/well unless otherwise specified. Plasmid DNA, peptide, and liposome solution were prepared in a transfection optimizing medium (TOM, Welgene Co., Korea) in 50 µl volume, and plasmid DNA was first mixed with the peptide. After 10 min incubation of the DNA:peptide complex, we mixed this complex with liposome and incubated it for 15 min more at room temperature. Twenty-four hours prior to transfection, the cells were transferred to 48-well culture plates at a density of 40,000 cells/well for MCF-7 and VSMC. Thirty minutes before transfection, the medium was removed and the cells from each well were briefly washed with 100 µl of sterile phosphate-buffered saline (PBS); then 150 µl of TOM was added to each well. The wells then received 150 µl of DNA:peptide:lipid complex, and the plates were incubated for 4 h. An additional 300 µl of medium (20% FBS) was added to each well so as to achieve a final serum concentration of 10%, and the plates were incubated for a further 24 h. Commercial liposomes were also tested in order to reveal the best conditions within the range of the manufacturer’s protocol, and used here in those optimal conditions.

**Luciferase Assay and GFP Expression**

Twenty-four hours after transfection, the medium was aspirated and the wells were washed twice with 200 µl of ice-cold PBS. To each well, 100 µl of 1× reporter lysis buffer (Promega Corp., USA) was added and the cells were lysed for 1 h in an ice tray. The cell lysates were completely collected into Eppendorf tubes and centrifuged (12,000 rpm, 4°C) for 5 min. The supernatant was transferred to

### Table 1. List of peptides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Number</th>
<th>Modification</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat</td>
<td>RKKRRQRRRPPQ</td>
<td>12</td>
<td></td>
<td>1,661</td>
</tr>
<tr>
<td>Tat-CTHD</td>
<td>RKKRRQRRRPPQC</td>
<td>26</td>
<td>C-Terminal disulfide homodimer</td>
<td>3,528</td>
</tr>
<tr>
<td>Tat-NTHD</td>
<td>CRKRRQRRRPPQ</td>
<td>26</td>
<td>N-Terminal disulfide homodimer</td>
<td>3,528</td>
</tr>
<tr>
<td>Cyclic-Tat</td>
<td>CRKRRQRRRPPQC</td>
<td>14</td>
<td>Disulfide bond between C-terminal and N-terminal</td>
<td>1,866</td>
</tr>
</tbody>
</table>