Tat Peptide-based Gene Delivery to Human Bone Marrow Stromal Cells as a Platform for Tissue Regeneration: In Vitro Study

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Bone marrow stromal cells (BMSC) hold promise for osteogenic differentiation and can be augmented by the application of genes encoding bone morphogenetic proteins (BMPs). Several gene delivery approaches have been employed to these tissue engineering attempts. Despite some successes of the gene delivery protocol, however, both virus-based delivery and cationic lipid based delivery system have been beset by the immune response as well as inflammatory reaction to these applied delivery carriers. An alternative approach involving complexation of gene products with cell penetrating peptides seems promising. Herein, plasmid DNA coding BMP was condensed with cell penetrating peptide, TAT, and examined internalization, gene expression level as well as differentiation in BMSC. Synthetically prepared TAT peptides were able to condense and form complex with the BMP-coding pDNA, and efficiently transferred the pDNA into nucleus and cytoplasm in a short time period. The produced amount of BMP from the BMSC was higher when cells were treated with TAT-pDNA complex thereby inducing marked mineralization of the cultures. Taken together, the present study suggested that cell penetrating peptide TAT could be a useful and safe tool for enhancing delivery of BMP gene into stem cells, which opens wide applicability in the regenerative therapeutic strategy.

Key words: Bone marrow stromal cells, Osteogenic differentiation, Cell penetrating peptide, TAT, Bone morphogenetic protein

INTRODUCTION

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tem cell-mediated gene therapy of growth factor including bone morphogenetic protein (BMP) has been one of the most attractive approaches for tissue regeneration procedure.1-4 In principle, a growth factor or other tissue inductive factor is produced by the cells that have been genetically modified ex vivo to express the desired gene. Using this type of gene therapy, mesenchymal stem cells (MSCs) have been genetically modified to express the several types of growth factors including BMP, transforming growth factor (TGF) and insulin like growth factor (IGF).5-8 Transducing BMP gene to MSCs has been a routine procedure to stimulate osteogenic differentiation in vitro. However, despite some success of the BMP gene delivery using viral vectors, employment of these vectors poses concerns related to immunogenicity and uncontrollable expression, and disturbance of the host genome.9 Delivery of plasmid DNA with nonviral carrier has been thus pursued as an alternative approach to circumvent these issues. Nonviral gene carriers such as cationic liposome, synthetic polymers are shown to introduce a significantly reduced immunogenicity than the viral vectors in vitro, but their in vivo applications are still hampered by the low transfection efficiency and sometimes the poor biocompatibility, as well as by the cytotoxicity of the polymeric carriers and/or their degradation products.10-13 The quest for an alternative gene carrier therefore remains in genetic modification of stem cells.

The authors recently reported the several transducing peptide carriers for effective gene and protein delivery into the cells.14,15 These peptides termed as protein transduction...
domain (PTD) or cell penetrating peptide (CPP), comprised a highly basic and hydrophilic peptide consisting of more than six arginine residues in their amino acid sequences. The examples of these peptides include transactivating transcriptional factor (TAT), Antennapedia peptide, protamine fragments. At a concentration as low as 0.1 mM the CPP peptide, for example, TAT was shown to translocate through the cell membranes within a period of 5 min. In addition, by genetic or chemical hybridization of this TAT peptide to a variety of biologically active species, including proteins, peptides, DNA, and even magnetic nanoparticles, this TAT peptide was shown to be able to facilitate a rapid internalization of all of such attached species into various cell types.

Hence, in the study reported here, we explored the possibility and potential of utilizing this TAT peptide as an effective carrier for gene delivery. The in vitro capability of TAT in cell trafficking, condensing DNA, translocating DNA inside the cells, protecting DNA from DNase I degradation, transfecting cells, and inducing osteogenic differentiation of the bone marrow stromal cells (BMSCs) was thoroughly examined.

**MATERIALS AND METHODS**

**Materials**

The 3-[(4,5-dimethylthiazol-2-yl) 25-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), 0.25% trypsin-EDTA, Roswell Park Memorial Institute medium (RPMI 1640), Dulbecco’s modified essential medium (DMEM), penicillin and streptomycin were purchased from Gibco-BRL (Gaithersburg, MD, USA). The BCA protein assay reagent was from Pierce (Rockford, IL, USA). The peptide containing the TAT (CYCGRKRKKRRQRRRRR) sequence was synthesized using F-moc chemistry based fully automated peptide synthesizer (APEX 396, AAPP TEC, Lousiville, KY, USA) and purified using HPLC. Hoechst 33342 dye was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Fluorescein-labeled plasmid DNA (pGeneGripTM) labeled plasmid DNA (pGeneGripTM) was obtained from Gene Therapy System Inc. (San Diego, CA, USA). All of the solvents used were analytical grade and water was distilled and deionized.

**Cell Culture**

Human bone marrow stromal cells (hBMSC) were plated in T75 flasks for continuous passaging in DMEM supplemented with 10% FBS (Gibco) and 1% antibiotic-antimycotic solution (Gibco). Medium was changed twice weekly and cells were detached by trypsin-EDTA and passed into fresh culture flasks at a ratio of 1:4 upon confluence. Cultures were incubated at 37°C under a humidified atmosphere containing 95% air and 5% CO₂.

**Preparation of Plasmid DNA/TAT Complexes**

The pDNA/TAT complexes coding BMP were prepared by mixing various amounts of TAT (increasing from 1-20 µg in 10 µl water) with plasmid (pCMV-cDNA3-BMP 1 µg in 10 µl water). The solution was allowed to stand for 30 min at 20°C for complex formation. By mixing with various amounts of TAT, the pDNA/TAT complexes comprising different charge ratios (+/−) ranging from 1:1 to 1:20 were obtained. Formation of the pDNA/TAT complexes was monitored by 1.0% agarose gel electrophoresis using DNA molecular markers. Following electrophoresis, the gels were stained with 0.5 µg/ml ethidium bromide for 45 min and analyzed on a UV illuminator to identify the locations of DNA. FITC-labeled pDNA/TAT complexes were prepared by mixing TAT with FITC-labeled pDNA (pGeneGripTM) at a charge ratio = (+/−) 1: 10. The solutions were incubated at 20°C for 30 min for complex formation.

**Stability of Plasmid DNA/TAT Complexes**

The charge ratio (+/−) used in the preparation of the pDNA/TAT complex was controlled at 1: 10. After complex formation, DNase I (50 units, Gibco BRL) was added to the complex suspension, and the solution was incubated at 37°C for 60 min. Naked pDNA was used as the control. At time intervals of 72, 10, 20, 40, and 60 min during incubation, 50 µl of the complex suspension were withdrawn, mixed with 75 µl of the stop solution (4 M ammonium acetate, 20 mM EDTA, and 2 mg/ml glycerol), and then placed on ice. The pDNA was dissociated from TAT by adding 37 µl 1.0% SDS to the complex suspension and then heating the mixture at 65°C overnight. The pDNA was extracted and precipitated by treating the solution mixture with phenol/chloroform and ethanol several times. The precipitated DNA pellet was then dissolved in 10 µl of TE buffer and subjected to 1.0% agarose gel electrophoresis.

**Confocal Laser Scanning Microscopy**

FITC-labeled free pDNA, DNA/TAT complex was added to BMSCs (10⁵ cells) cultured in Lab-Tek chambered slides. After 1 h incubation, cells were washed with PBS and further incubated with 50 ng/ml Hoechst 33342 dye in PBS supplemented with 1% FBS at room temperature for 15 min. Cells were then washed with PBS, and fixed with 1% paraformaldehyde for 20 min. The fixed cells were washed again and mounted with PBS/glycerol containing antifading agent. Confocal laser scanning microscopy was carried out using an inverted LSM 510 laser scanning microscope (Carl Zeiss, Gottingen, Germany) equipped with a Plan-Apochromat 63×1.4 N.A. or 40×1.4 N.A. lens. The laser was set at 488 nm (blue) and 543 nm/594 nm to produce the excitation wavelengths for fluorescein and rhodamine, respectively. Hoechst dye fluorescence was collected by the laser tuned to 790 nm/400 nm for two-photon excitation of the dye. Z-series
were taken of a 1 to 2 micron optical section at 2-µm intervals.

**Flow Cytometric Analysis**

The BMSC cells were seeded at a density of $1 \times 10^6$ cells per well in 6-well plates in 1.5 ml culture medium. One day later, the cells were washed and incubated with FITC-labeled peptides for 0.5, 1, 4, 10 and 24 h. For studies related to the complexes, FITC-labeled pDNA, or pDNA/TAT complex was incubated with the above BMSCs for 1 h. After incubation, the cells were washed with PBS, trypsinized, and washed. The cells were then fixed with 1% paraformaldehyde and washed with PBS. Analysis was conducted on a FACScaliber flow cytometer (Beckton Dickinson, San Jose, CA, U.S.A.) equipped with a 488 nm air-cooled argon laser. The filter settings for emission were 530/30 nm bandpass (FL1) for FITC. The fluorescence of 10,000 vital cells was acquired and data was visualized in logarithmic mode.

**In Vitro Quantification of rhBMP-2 Expression**

To quantify the secretion of recombinant human bone morphogenetic protein (rhBMP-2), human bone marrow stromal cells (hBMSCs) ($5 \times 10^5$ cells) were treated with TAT peptide hybrids with 5 mg plasmid and cultured for 24 h. Medium from the transfected hBMSCs was then collected and used for an enzyme-linked immunosorbent assay (ELISA). An ELISA for hBMP-2 was performed using the Quantikine hBMP-2 Immunoassay kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) according to the manufacturer’s protocol.

**In Vitro Mineralization**

Human bone marrow stromal cells (hBMSCs) were cultured in α-MEM containing 15% fetal bovine serum, 1% antibiotic-antimycotic solution, 10 mm sodium β-glycerol phosphate, 50 μg/ml l-ascorbic acid, and 10-7 M dexamethasone. The pDNA coding BMP-2, either with TAT complex or alone, were applied to cell cultures at a DNA equivalent concentration of 5 μg and further incubated for 10 days. The culture was supplemented with 1 mg of calcine during the observation period for ease of detecting mineralized extracellular matrix in the cells under a confocal laser microscope (FV-300, Olympus Co., Tokyo, Japan). In addition, mineralized extracellular matrix was observed by staining with Alizarin-Red S in a separate set of cultures. Cells were washed, fixed with 95% ethanol at 4°C for 15 min, then stained with 2% Alizarin-Red S solution for 10 min. Unbound stain from the culture was removed, and the red-stained mineralized matrix surface was photographed with an Olympus inverted microscope.

**Statistical Analysis**

All values are presented as mean ± SE for all controls (DNA alone) and experiments (DNA complex with TAT, N=total number of independent cultures). Data were analyzed by one-way ANOVA followed by Fisher’s protected least significant difference (PLSD) posthoc test (StatView; SAS Institute, Cary, NC, USA). P values of < 0.05 were considered significant.

**RESULTS AND DISCUSSION**

**Complex Formation and Stability of pDNA/TAT Complexes**

A gel retardation study was performed to confirm the formation of complexes between TAT and pDNA. One microgram of plasmid was mixed with various amounts of TAT to yield different charge ratios (-/+) between pDNA and TAT. The pDNA/TAT complexes were analyzed by 1.0% agarose gel electrophoresis. As seen in Figure 1A, the pDNA/TAT complexes were completely retarded in their migration at the 1 : 2 charge ratio (-/+) between pDNA and TAT. These results suggested that TAT was able to effectively condense plasmid DNA into complex at the charge ratio of 1 : 2 (-/+), as the migration of the formed pDNA/TAT complex on agarose gel was considerably retarded due to charge neutralization and the increase in the overall molecular size. The charge ratio of complex using TAT is somewhat higher than that from our previous results using other peptides. Previous our results

![Figure 1](image-url)
using 10 arginine containing protamine fragment termed as LMWP formed complex with DNA at a charge ratio of 1:1, which makes sense, as LMWP has higher arginine content which leads easy formation of complex.\textsuperscript{14} However, these findings are in good agreement with those observed by previous other reports, indicating that TAT formed a condensed complex with pDNA as effectively as any of the currently used cationic polymeric gene carriers.\textsuperscript{10-12}

Since pDNA can be easily degraded by nucleases in the cytosol, instability of pDNA may remain one of main obstacles for the delivery of therapeutic genes in genetic modification of stem cells. DNase I is known to be a major nuclease present in the serum or cytosol. Therefore, for a successful gene delivery, it is of important to protect DNA from degradation by DNase I. To demonstrate that TAT could protect pDNA from nuclease degradation, the DNase I protection assay was conducted by incubating the plasmid/TAT complex with DNase I. As shown by the results from agarose gel electrophoresis (Figure 1B), naked DNA was destroyed after 10 min of incubation with DNase I (Figure 1B, left column). However, pDNA complex with TAT at a charge ratio 1:4 (−/+ ) was not destroyed after more than 60 min of incubation with DNase I (Figure 1B, right column). Therefore, it was clear that TAT peptide could protect plasmid DNA from the degradation by DNase I.

**Intracellular Uptake of pDNA/TAT Complexes**

The TAT peptide was evaluated for its ability to facilitate cellular pDNA delivery when forming the condensed complex. FITC-labeled pDNA was mixed with TAT with a charge ratio (−/+ ) = 1:10. Our previous result indicated gene expression was highest at a charge ratio of 1:10 (−/+ ),\textsuperscript{14} therefore, we selected the complex with the charge ratio of 1:10 in this study. As shown in Figure 2A, there was virtually no cellular uptake of free FITC-labeled pDNA. In contrast, incubation of TAT with FITC-pDNA promoted internalization of FITC-labeled pDNA in the cells; with a large fraction of pDNA being seen to localize in the nucleus (Figure 2B). For TAT complexes, fluorescein was visualized in both the nucleus and cytoplasm. Superimposition of the two images by fluorescein and Hoechst 33342 dye (for nuclear staining) clearly demonstrated a nuclear localization of the pDNA. As reported previously, TAT can facilitate the delivery of its associated molecules into cells in a short time period.\textsuperscript{15–18} Confocal microscopy data in combination with the flow cytometric analysis (Figure 2C) confirmed that TAT mediated pDNA cell internalization within 1 h. Overall, results from the cell uptake studies clearly demonstrated that TAT could effectively deliver pDNA in the cytosol by forming a complex.

**In Vitro BMP Expression and Osteogenic Differentiation by pDNA/TAT Complex**

To estimate the efficiency of protein synthesis after BMP-2 gene transfer by complex with TAT peptide, immunoassay of media collected from culture of transduced BMSCs was con-
ducted. Human BMSCs secreted rhBMP-2 at levels ranging from 3.2 to 7.4 ng/10^6 cells in 24 h (Figure 3A), whereas BMSCs treated naked gene secreted levels of only 0 to 0.3ng/10^6 cells in 24 h. Therefore, peptide transduction of BMSCs with BMP-2 gene resulted 10 fold increased in BMP-2 secretion. The level of BMP-2 measured in our experiments is somehow approaching to similar level to values reported by others for viral gene delivery of BMP-2^2^,4). Based on these studies and our data, we next evaluated whether the levels of BMP-2 secretion in our experiment is sufficient to induce osteogenic differentiation. In vitro matrix mineralization was examined as an end-point indicator of the osteoblastic phenotype and the ultimate demonstration of the activity of BMP-2. Enhanced mineralization was seen for BMSCs transduced pDNA/TAT complexes, in a sharp contrast to either that cultured with plasmid alone or untreated cells (Figure 3B). It should be noted that peptide complex was able to transduce plasmid DNA and thereby induce BMSC differentiation. Taken together, cell penetrating peptide such as TAT was able to condense therapeutic gene and form a stable complex, translocate efficiently into the stem cells. The BMP gene expression was significantly higher when stem cells were treated with the pDNA complex with TAT peptide and thus induce marked osteogenic differentiation. A further experiments regarding the duration of gene expression, molecular signal change inside the BMSCs by the transfection of BMP gene, together with gene expression by the complex in vivo need to be conducted and are now process in our laboratory.

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