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Controlled release of cell-permeable gene complex from poly(L-lactide) scaffold for enhanced stem cell tissue engineering

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ABSTRACT

The use of tissue engineering to deliver genes to stem cells has been impeded by low transfection efficiency of the inserted gene and poor retention at the target site. Herein, we describe the use of non-viral gene transfer by cell-permeable peptide (CPP) to increase the transfection efficiency. The combination of this technique with the use of a controlled release concept using a poly (L-lactide) scaffold allowed for prolonged uptake in stem cells. High transfection efficiency was obtained using a human-derived arginine-rich peptide denoted as Hph-1 (YARVRRRGPRR). The formation of complex between pDNA and Hph-1 was monitored using gel retardation tests to measure size and zeta potential. Complex formation was further assessed using a DNase I protection assay. A sustained gene delivery system was developed using a fibrous 3-D scaffold coated with pDNA/Hph-1 complexes. Transfection efficiency and the mean fluorescence intensity of human adipose-derived stem cells (hASCs) on the sustained delivery scaffold were compared to those of cells transfected via bolus delivery. Plasmid DNA completely bound Hph-1 at a negative-to-positive (N/P) charge ratio of 10. After complex formation, Hph-1 appeared to effectively protect pDNA against DNase I attack and exhibited cytotoxicity markedly lower than that of the pDNA/PEI complex. Plasmid DNA/Hph-1 complexes were released from the scaffolds over 14 days and were successfully transfected hASCs seeded on the scaffolds. Flow cytometry revealed that the transfection efficiency in hASCs treated with pDNA/Hph-1 complex was approximately 5-fold higher than that in cells transfected using Lipofectamine. The sustained delivery system showed a significantly higher transfection efficiency and remained able to transfect cells for a longer period of time than bolus delivery. These results suggest that cell-scaffold-based tissue regeneration can be further improved by transduction concept using CPP and controlled release using polymeric scaffold.

1. Introduction

Stem cell transplantation represents a promising means for the repair of defective tissues. Recent studies have indicated that adipose-derived stem cells (ASCs) possess at least trilineage potential (bone, cartilage, and fat) and can be classified as stem cells [1–5]. The extensive proliferation and multilineage differentiation capabilities of ASCs provide an attractive opportunity for stem cell-based gene therapies. ASCs are easier to obtain and are more abundantly available than bone marrow stem cells (BMSCs). The ASC yield from adipose tissue is approximately 40-fold higher than the stem cell yield from bone marrow [6]. ASCs can also be induced to express genes and protein markers associated with chondrocytes, myocytes, adipocytes, or osteoblasts [7–10]. ASCs are thus considered ideal candidates for use in stem cell-based gene therapy; however, stem cells including ASCs, have been resistant to non-viral transfection methods such as lipofectants [11]. Most current methods for genetic modification of stem cells utilize modified viruses, such as adenovirus, lentivirus and retrovirus [12–14]. Although these viral vectors may effectively transfer DNA to stem cells, concerns related to safety issues, including immunogenicity, toxicity and mutagenesis remain. In contrast, non-viral vectors offer safety advantages over viral vectors due to their low immunogenicity and toxicity, as well as their ease of production [15]. Despite these advantages, existing non-viral vectors including polymer-based gene delivery systems present significantly lower transfection efficiency and thus might not be able to induce stem cell gene transfection [16]. For these reasons, potential biocompatible carrier for therapeutic genes to stem cells is needed.

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Cell-permeable peptides (CPP) are short peptides that can penetrate plasma membranes and deliver a variety of molecules in cells [17–20]. In a previous study, we identified a novel CPP (YARVRRRGPRR) from the human transcription factor Hph-1 and demonstrated its powerful transduction ability *in vitro* and *in vivo* [21]. The Hph-1 protein formed a complex with plasmid DNA through the specific interaction between GAL4–DBD and UAS, which was then able to deliver genes into the cells via Hph-1. Therefore, we anticipated that Hph-1 may be used to overcome the low efficiency of stem cell transfection.

In tissue engineering, growth factor signaling is required over a span of weeks; therefore, expression of a delivered gene is also required for intervals lasting weeks. Further, gene expression is typically only desired at the site at which tissue formation is required. Biodegradable polymers acting as DNA reservoirs can offer localized delivery of DNA in a controlled fashion [22–24]. Previous studies have shown that sustained delivery of plasmid DNA from polymer matrices led to long-term transfection of large numbers of cells both *in vitro* and *in vivo* [25–27]. The released DNA transfected seeded cells to produce gene products or biological signals locally and in a sustained manner. Directly delivered naked plasmids are rapidly degraded by DNase and cleared from the tissue [28]. The sustained release system can protect DNA from nucleases and can replace DNA that is cleared or degraded, thereby maintaining consistent effective concentration levels within the tissue [29]. The objective of this approach is to maintain the DNA at or near the surface of the scaffold in order to promote internalization by cells that adhere to the scaffold. This sustained release system provides several pharmacological advantages, such as a prolonged transfection period, increased stability, and superior targeting of the DNA. Thus, transfection of adhered cells can be enhanced by sustained release of the DNA and immobilization of DNA in contact with adherent cells.

Herein, the *in vitro* capability of Hph-1 to condense DNA, translocalize DNA inside the hASCs, protect DNA from DNase I degradation, and induce cytotoxicity in the cells will be discussed in detail. After pDNA/Hph-1 complexes were deposited directly onto the scaffold, the release of pDNA from the scaffold and the transfection potential of the pDNA/Hph-1 complexes were examined. The characteristics of the newly developed gene delivery system were evaluated in comparison to the bolus delivery system. In addition, the effect of sustained release on gene expression will be discussed in this paper.

2. Materials and methods

2.1. Materials

Poly (L-lactic acid) (PLLA) (MW 370,000 Da) and poly (DL-lactide-co-glycolide) (PLGA) (50:50 monomer molar ratio) (MW = 130,000) were provided by Purac Biochem BV (Gorinchem, Holland). DMEM, trypsin–EDTA, fetal bovine serum (FBS), antibiotics, antimycotics and Opti-Minimum Essential Medium (Opti-MEM) were purchased from Gibco (Grand Island, USA). Lipofectamine™ 2000 and DAPI were purchased from Invitrogen Co. (Carlsbad, USA). Polyethyleneimine (PEI) (branched, MW = 25,000) was obtained from Polyscience Co. (St. Louis, USA). All solvents used were of analytical grade.

2.2. Human adipose-derived stem cell isolation and characterization

Adipose tissue-derived mesenchymal stem cells (ASCs) were isolated from freshly excised human subcutaneous fat tissue. After mincing into pieces of 2–3 mm^3 size, the adipose tissue was washed with Dulbecco's Phosphate Buffered Saline (DPBS). Adipose tissue was digested with 0.05% collagenase type I (Sigma Chemical Co., St. Louis, USA) and 1% BSA dissolved in collagenase buffer (100 mM HEPES, 120 mM NaCl, 50 mM KCl, 1 mM $CaCl_2$, 50 mM glucose, pH 7.4) for 30 min at 37 °C under constant shaking. The tissue slurry was filtered through a 250 μm filter and centrifuged at 500×*g* for 20 min at room temperature. After the supernatant was discarded, the cell pellet was resuspended in DMEM supplemented with 10% FBS. Cells were grown to subconfluence, trypsinized after washing, and subcultured. ASCs were used at passage six for this experiment. For characterization, 10^6 hASCs were washed with PBS and incubated with fluorescently labeled antibodies (1 μg/10^6 cells) for CD14, CD29, CD31, CD34, CD44, CD45, CD90 and CD105 (BD Pharmingen, San Diego, USA) in PBS, pH 7.5 supplemented with 1% bovine serum albumin (BSA) for 30 min on ice; they were then washed with PBS and fixed in 1% paraformaldehyde (R&D System, Minneapolis, USA) solution. Cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences). At least 10,000 events were acquired per sample. Data acquisition and analysis were performed using CellQuest Pro software.

2.3. Preparation of pEGFP/Hph-1 complexes

The reporter gene pEGFP, which contains GAL4-specific recognition sites (UAS) and the gene encoding enhanced green fluorescent protein was constructed using the plasmid pEGFP-N1 (Clontech). The GAL4-specific sites were amplified from the pG5CAT vector (Clontech), which contains five consensus GAL4 binding sites (UAS_{17mer}x5). *E. coli* BL21 star (DE3) pLysS cells (Invitrogen) carrying pHph-1–Gal4–DBD were grown at 37 °C to $OD_{600} = 0.6$ in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Protein expression was induced for 4 h at 37 °C by adding 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, resuspended in lysis buffer (10 mM imidazole, 50 mM sodium phosphate and 300 mM NaCl, pH 8.0) and lysed by sonication. Lysates were clarified by centrifugation (500×*g* for 20 min at 4 °C) and gently mixed with a 50% slurry of Ni-NTA resin (Qiagen, Hilden, Germany) for 1 h at 4 °C. Unbound proteins were removed by washing the column with 20 mM imidazole buffer, and bound proteins were eluted with 250 mM imidazole buffer. The proteins were pooled, dialyzed against Phosphate Buffered Saline (PBS) with 10% glycerol and stored at 80 °C before use.

The TAT protein was constructed and purified as a typical PTD for comparison of transfection efficiency with Hph-1. pEGFP/Hph-1, pEGFP/TAT and pEGFP/PEI complexes were prepared by mixing Hph-1, TAT or PEI, respectively, with pEGFP at various charge ratios. The solutions were incubated for 30 min at room temperature for complex formation. By mixing these pEGFP complexes with various amounts of CPPs, pEGFP/carrier complexes comprising different charge ratios (−/+) ranging from 1:1 to 1:10 were obtained. Formation of the pEGFP/Hph-1 complexes was monitored by 1.0% agarose gel electrophoresis. The particle size and zeta potential of pEGFP/Hph-1 complexes with charge ratios from 1:1 to 1:10 (−/+) were determined by dynamic light scattering (DLS, ELS-8000, Photal, Japan) at room temperature.

2.4. Stability of the pEGFP/Hph-1 complexes

The charge ratio (−/+) used in the preparation of the pEGFP/Hph-1 complex was controlled at 1:10. After complex formation, DNase I (50 U, SIGMA) was added to the complex suspension, and the solution was incubated at 37 °C for 60 min. Naked pEGFP was used as the control. At time intervals of 0, 10, 20, 40, 60, and 80 min during incubation, 50 μl of the complex suspension was withdrawn, mixed with 75 μl of the stop solution (4 M ammonium acetate, 20 mM EDTA, and 2 mg/ml glycogen), and then placed on ice. The pEGFP was dissociated from Hph-1 by adding 37 μl 1.0% SDS to the complex suspension and then heating the mixture at 65 °C overnight. The pEGFP was extracted and precipitated by treating the solution mixture with phenol/chloroform and ethanol several times. The