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The enhancement of bone regeneration by gene activated matrix encoding for platelet derived growth factor

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ABSTRACT

Gene therapy using non-viral vectors that are safe and efficient in transfecting target cells is an effective approach to overcome the shortcomings of protein delivery of growth factors. The objective of this study was to develop and test a non-viral gene delivery system for bone regeneration utilizing a collagen scaffold to deliver polyethylenimine (PEI)-plasmid DNA (pDNA) [encoding platelet derived growth factor-B (PDGF-B)] complexes. The PEI-pPDGF-B complexes were fabricated at amine (N) to phosphate (P) ratio of 10 and characterized for size, surface charge, and *in vitro* cytotoxicity and transfection efficacy in human bone marrow stromal cells (BMSCs). The influence of the complex-loaded collagen scaffold on cellular attachment and recruitment was evaluated *in vitro* using microscopy techniques. The *in vivo* regenerative capacity of the gene delivery system was assessed in 5 mm diameter critical-sized calvarial defects in Fisher 344 rats. The complexes were ~100 nm in size with a positive surface charge. Complexes prepared at an N/P ratio of 10 displayed low cytotoxicity as assessed by a cell viability assay. Confocal microscopy revealed significant proliferation of BMSCs on complex-loaded collagen scaffolds compared to empty scaffolds. *In vivo* studies showed significantly higher new bone volume/total volume (BV/TV) % in calvarial defects treated with the complex-activated scaffolds following 4 weeks of implantation (14- and 44-fold higher) when compared to empty defects or empty scaffolds, respectively. Together, these findings suggest that non-viral *PDGF-B* gene-activated scaffolds are effective for bone regeneration and are an attractive gene delivery system with significant potential for clinical translation.

1. Introduction

Identification of key molecules involved in bone formation and fracture healing has led to the development of biomimetic materials for clinical applications [1,2]. One such development in dentistry is the introduction and usage of recombinant growth factors and morphogenetic proteins [3]. Major barriers with protein therapy are cost, low bioavailability and supraphysiological dosage for therapeutic efficacy [4]. One strategy to overcome these drawbacks is gene therapy [5,6]. There are two primary methods of gene therapy for bone regeneration: 1) transfection of cells *in vitro* and subsequent transplantation into the site of the bone defect [7], and 2) direct delivery of osteogenic plasmid genes immobilized in a scaffold matrix [8]. The latter approach has been shown to be more advantageous in generating a persistent expression of the growth factors by the transfected wound repair cells, more cost-effective, and may be more clinically safe for use [8–11].

The first set of *in vivo* studies involving non-viral gene activated matrices for bone regeneration utilized plasmids encoding bone morphogenetic protein-2 (BMP-2) and/or human parathyroid hormone peptide [8,9]. Non-viral gene delivery vectors are relatively safe compared to viral vectors but have lower transfection efficiencies that

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can often limit their potential [12]. One non-viral gene delivery system showing strong transfection capabilities is cationic polymer, polyethylenimine (PEI). In previous studies, the branched form of PEI has shown significantly higher gene transfer efficiency than the linear form of PEI [13]. Branched PEI exhibits considerable buffering capacity over a wide pH range due to its protonability, has the highest cationic-charge potential, and condenses plasmid DNA (pDNA) to a greater extent than the linear PEI. This protects the DNA from serum DNases, cytosolic nuclease digestion, facilitates endocytosis and promotes the 'proton sponge effect' [14–17]. Different molecular weights of branched PEI have been investigated *in vivo* for their transfection efficiencies with 25 kDa PEI yielding the highest transfection efficiency. Low molecular weight PEIs resulted in weak PEI-pDNA complexes that readily dissociated, thus reducing the transfection efficiency relative to 25 kDa PEI [18–21].

Platelet derived growth factor (PDGF) is a potent mitogen and chemoattractant for mesenchymal and osteogenic cells and a stimulant for the expression of angiogenic molecules that play a pivotal role in bone healing [22]. There are several preclinical and clinical reports that have shown the safety and efficacy of PDGF in achieving bone regeneration [23–25]. Past studies on the use of PDGF have been through viral vector delivery or as a recombinant protein [23–25]. The objective of this study was to develop, optimize and test a non-viral based gene delivery system for bone regeneration utilizing a collagen scaffold loaded with cationic PEI-pDNA [encoding PDGF-B] complexes.

2. Materials and methods

2.1. Materials

Branched PEI (mol. wt. 25 kDa) was purchased from Sigma–Aldrich® (St. Louis, MO). The GenElute™ HP endotoxin-free plasmid maxiprep kit was obtained from Sigma–Aldrich®. The luciferase assay system was purchased from Promega Corporation (Madison, WI). The microBCA™ protein assay kit was purchased from Pierce (Rockford, IL). The PDGF-BB ELISA kit was purchased from Quantikine® (R & D Systems®, Minneapolis, MN). Plasmid DNA (6.4 Kb) encoding for firefly luciferase reporter protein (pLUC) driven by cytomegalovirus (CMV) promoter/enhancer (VR1255 pDNA) was obtained from *Vical*®, Inc. (San Diego, CA). Plasmid DNA (4.7 Kb) coding for the enhanced green fluorescent protein (pEGFP-N1) driven by CMV promoter/enhancer was obtained from Elim Biopharmaceuticals, Inc. (Hayward, CA). Plasmid DNA (4.9 Kb) encoding PDGF-B protein (pPDGF-B) driven by CMV promoter/enhancer was obtained from Origene Technologies, Inc. (Rockville, MD). Absorbable type-I bovine collagen was obtained from Zimmer Dental Inc. (Carlsbad, CA). All other chemicals and solvents used were of reagent grade. Human bone marrow stromal cells (BMSCs) and Dulbecco's modified eagle medium (DMEM) were purchased from American Type Culture Collection (ATCC®, Manassas, VA). Trypsin-EDTA (0.25%, 1X solution) and Dulbecco's phosphate buffered saline (PBS) was purchased from Gibco® (Invitrogen™, Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals® (Lawrenceville, GA). Gentamycin sulfate (50 mg/ml) was purchased from Mediatech Inc. (Manassas, VA). MTS cell growth assay reagent (Cell Titer 96® AQueous One Solution cell proliferation assay) was purchased from Promega Corporation. Alexa Fluor® 568 phalloidin was purchased from Invitrogen. Triton X-100 was obtained from Sigma–Aldrich®. Vectashield®, Hardset™ mounting medium with 4′,6-diamidino-2-phenylindole (DAPI, H-1500) was obtained from Vector Labs Inc. (Burlingame, CA).

2.2. Preparation of pDNA encoding different proteins: pLUC, pEGFP-N1 or pPDGF-B

The chemically competent DH5α™ bacterial strain (*Escherichia coli* species) was transformed with pDNA to amplify the plasmid. The pDNA in the transformed cultures was then expanded in *E. coli* in Lennox L Broth (LB Broth) overnight at 37 °C in an incubator shaker at 300 rpm. Plasmid DNA was extracted using GenElute™ HP endotoxin-free plasmid maxiprep kit and was analyzed for purity using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermoscientific, Wilmington, DE) by measuring the ratio of absorbance (A260 nm/A280 nm). The concentration of pDNA solution was determined by absorbance at 260 nm.

2.3. Fabrication of PEI-pDNA complexes

Complexes were prepared by adding 500 μl PEI solution drop wise to 500 μl pDNA (pLUC/pEGFP-N1/pPDGF-B) solution containing 50 μg pDNA and mixed by vortexing for 20 s. The mixture was incubated at room temperature for 30 min to allow complex formation between the positively charged PEI (amine groups) and the negatively charged pDNA (phosphate groups) [16,26]. Complexes were fabricated using different N (nitrogen) to P (phosphate) ratios (molar ratio of amine groups of PEI to phosphate groups in pDNA backbone) by varying the PEI amounts and maintaining the amount of pDNA constant (N/P ratios of 5, 10, 15 and 20, Table 1). Final volume of the complexes used in the transfection and cytotoxicity experiments was 20 μl containing 1 μg of pDNA.

2.4. Size and surface charge of the PEI-pPDGF-B complexes

Measurements were carried out using a Zetasizer Nano-ZS (Malvern Instruments, Westborough, MA). The particle size and size distribution by intensity was determined by dynamic laser light scattering (4 mW He–Ne laser with a fixed wavelength of 633 nm, 173° backscatter at 25 °C) in 10 mm diameter cells. Surface charge (zeta potential) was measured electrophoretically by the laser scattering technique using folded capillary cells. All measurements were done in triplicate. The mean value was recorded as the average of three different measurements.

2.5. Cell culture

Human BMSCs were maintained in DMEM supplemented with 10% FBS and 50 μg/ml gentamycin in a humidified incubator at 37 °C containing 95% air and 5% CO_2 (Sanyo Scientific, Wood Dale, IL). Cells were grown as a monolayer on 75 cm^2 polystyrene cell culture flasks (Corning Incorporated, Corning, NY) and subcultured (subcultivation ratio of 1:9) after 80–90% confluence. Cell lines were started from frozen stocks and the medium was changed every 2–3 days. Cell passage numbers used in the experiments were between 4 and 10.

2.6. In vitro evaluation of the transfection efficiency of PEI-pLUC complexes in BMSCs

The PEI-pLUC complexes were prepared using N/P ratios of 1, 5, 10, 15 and 20. Cells were seeded at a density of 80,000 cells/well in 24-well plates (Costar®, Corning Inc, NY). The next day, at ~80% cell confluence, the cell culture medium was changed to serum-free medium and the treatments were gently vortexed and added drop wise into the wells. Each well was treated with 20 μl complexes containing 1 μg pLUC. Untreated cells were the controls while cells treated with PEI alone were the negative controls. Cells treated with uncomplexed pDNA served as a control comparison with complex-treated cells. Complexes were incubated with cells for 4 h or 24 h. At the end of each treatment period, cells were washed with 1X phosphate buffered saline (PBS) followed by addition of fresh complete medium. After a total incubation time of 48 h, cells were washed with 1X PBS, and treated with 1X lysis buffer and subjected to two freeze–thaw cycles whereupon cells were scraped and centrifuged at 14,000 rpm for 5 min. Luciferase expression was detected by a standard luciferase assay system. The relative light units (RLU) values per mg of the total cell protein, indicative of the transfection efficiency, were normalized against the protein concentration in cell extracts using a microBCA protein assay kit. The values are expressed as mean ± SD for each treatment ($n = 3$).

2.7. In vitro evaluation of toxicity of PEI-pLUC complexes in BMSCs

Cell survival assays were conducted to demonstrate the effect of N/P ratio of the PEI-pLUC complexes on the biocompatibility of complexes in BMSCs. Cells were seeded in clear polystyrene, flat bottom, 96-well plates (Costar®, Corning Inc.) at a density of 10,000 cells/well and allowed to attach overnight and further processed as described in Section 2.6. Untreated BMSCs were used as controls. Cells treated with PEI alone or uncomplexed pLUC alone served as additional controls. The complexes were incubated with the cells for 4 h or 24 h to mimic the conditions used in the transfection experiments. At the end of the incubation period, the cells were washed with 1X PBS and fresh complete medium was added to the cells followed by addition of 20 μL MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell growth assay reagent. The plates were then incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 h. The amount of soluble formazan produced by reduction of MTS reagent by viable cells was measured spectrophotometrically using SpectraMax® Plus^{384} (Molecular Devices, Sunnyvale, CA) at 490 nm. The cell viability was expressed by the following equation: cell viability (%) = (absorbance intensity of treated cells/absorbance intensity of untreated cells (control)) × 100. Values are expressed as mean ± SD for each treatment performed in triplicate.

Table 1
PEI amount in different N/P ratios used for formulating PEI-pDNA complexes (using 1 μg pDNA).

N/P ratio	PEI amount (μg)
1	0.13
5	0.65
10	1.30
15	1.95
20	2.6